Functional Studies of Vertebrate β-defensins

Natalie Louise Reynolds

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Declaration

I declare that the research and analysis presented in this thesis is my own unless otherwise stated. This work has not been submitted for any other degree and this thesis was composed entirely by myself.

Natalie L. Reynolds

March 2011

Abstract

The β-defensins are a family of small, cationic antimicrobial peptides. They are conserved in a variety of species ranging from lower vertebrates to mammals and plants, and were first identified for their broad-spectrum antimicrobial activity. Since their initial discovery, this role has broadened to include a number of diverse additional functions including the chemotaxis of immune cells (immature dendritic cells, macrophages and CD4+ T cells), the determination of coat colour in dogs and seed maturation in tomatoes.

The aim of this thesis is to investigate the function of vertebrate beta-defensins. I address this question by assessing how mouse β-defensin structure affects its bactericidal activity and also by carrying out β-defensin knockdown studies in zebrafish.

In this work, I address the question of how β-defensin structure affects its bactericidal activity by examining the effects of sequentially removing amino acids from the N-terminal of murine Defb14. I show that these deletions reduce bactericidal activity however the effects are much more striking in gram positive species than gram negative. Through the comparison of monomeric and dimeric species as well as analysis of peptide charge and hydrophobicity, this work indicates that a combination of primary sequence and structure is responsible for the bactericidal properties of this peptide.

In addition, this thesis describes the characterisation of three β-defensin-like peptides (Defbl1, Defbl2 and Defbl3) previously described in zebrafish. I utilise antimicrobial assays to determine the bactericidal properties of synthetic Defbl2 and Defbl3 against a panel of microbes and also show that Defbl1 is antimicrobially inactive. Furthermore, this work identifies the expression of *defbl1* during zebrafish gastrulation and utilises a combination of wholemount in situ hybridisation, morpholino knockdown and rescue and microarray analysis to ascertain a novel essential role for this peptide in early development.

This work is the first report of a β-defensin being involved in vertebrate development and presents a further widening of the influence of the defensin family.

Acknowledgements

Firstly, I would like to thank Dr. Julia Dorin and Dr Liz Patton for giving me the opportunity to start up this project and for giving me the freedom to come up with my own ideas and investigate them freely. Your trust and guidance has been invaluable.

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Thanks to Mum, Dad, George and Jan for pretending to find morpholinos fascinating and for taking an interest in everything that I do. Also to my grandparents Brenda and George Reynolds – I hope that wherever you are, you're proud of this latest "-ology".

Finally, thanks to Graeme – for so much. For learning more biology than you ever really wanted to know, for your endless patience, for being a personal 24hr taxi and unpaid slave to science (and me!). This thesis would have been possible without you, but I might not have come out with my sanity intact without you. This book is for you.

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Abbreviations

α-MSH	α-melanocyte stimulating hormone
°C	degrees celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CNV	copy number variation
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dpf	days post fertilisation
DTT	dithiothreitol
EARSS System	European Antimicrobial Resistance Surveillance
EDTA	ethylenediamine tetraacetic acid
EST	expressed sequence tag
FCS	foetal calf serum
g	gram

GFP	green fluorescent protein
HNP	human neutrophil peptide
hpf	hours post fertilisation
HSV	herpes simplex virus
IL1β	interleukin 1β
kb	kilobase
l	litre
LAP	lingual antimicrobial peptide
LB	Luria broth
LPS	lipopolysaccharide
Mb	megabase
MBC	minimum bactericidal concentration
MBT	midblastula transition
MIC	minimum inhibitory concentration
μM	micromolar
μm	micrometre
mM	millimolar
mRNA	messenger RNA
MRSA	methicillin-resistant *Staphylococcus aureus*
NaCl	sodium chloride
PBS	phospho-buffered saline
PFA	paraformaldehyde

PMN	polymorphonuclear leukocytes
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean
TAP	tracheal antimicrobial peptide
TB	tuberculosis
TLR	Toll-like receptor
TNFα	tumour necrosis factor α
UTR	untranslated region
UV	ultraviolet
VRSA	vancomycin-resistant *Staphylococcus aureus*
v/v	volume per volume
WHO	World Health Organisation
WISH	wholemount in situ hybridisation
w/v	weight per volume
XDR-TB	extensively drug resistant tuberculosis
ZFIN	Zebrafish Information Network

Amino Acid Single Letter Codes

A	Alanine
R	Arginine
N	Aspargine
D	Aspartate
C	Cysteine
Q	Glutamine
E	Glutamate
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

Chapter 1: Introduction

"I have been trying to point out that in our lives chance may have an astonishing influence and, if I may offer advice to the young laboratory worker, it would be this – never to neglect an extraordinary appearance or happening"

Sir Alexander Fleming

1.1 THE IMPORTANCE OF ANTIMICROBIAL AGENTS

A complex relationship exists between higher organisms and the microbial world. Shortly following birth, mammalian bodies are rapidly colonised by an array of bacteria to form a lifelong symbiotic relationship that benefits both the host and microbes. Recent data has shown that commensal bacteria of the *Bifidobacterium* genus confer protection against *E.coli* infection in mice (Fukuda *et al.*, 2011), illustrating the critical role commensals play in host defence mechanisms.

However, despite the benefits commensal bacteria confer, an intricate host innate immune barrier is essential to ensure that these colonising bacteria do not cross host epithelial linings to cause systemic infections. In addition, there is a limit to the number of colonies a host is able to support, and so colony numbers must also be kept under control.
In healthy humans the control of commensal colonies is rarely problematic, however in immune-challenged patients these bacteria can rapidly overcome host defence efforts and lead to opportunistic and nosocomial (hospital-acquired) infections. It is important that in such circumstances we have a battery of antibiotic agents with which to treat these infections otherwise the prognosis for these patients is poor. Some data have suggested that some 70% of hospital-acquired infections in the USA require some form of antibiotic treatment (Coates *et al.*, 2002).

Beyond the issue of control of commensals, antimicrobial agents have had a defining role throughout recent human history, proving to be vital in the control of diseases such as pneumonia, tuberculosis and systemic bacteraemia. In particular, the mortality rates of community-acquired bacterial meningitis strikingly demonstrate the impact antibiotics have had on the disease. As illustrated in Figure 1.1, meningitis mortality rates at the beginning of the century ranged from 80-100%, but the introduction of multiple antibiotics across the course of the century led to this dropping to a level of 7-30% in 2000 (Swartz, 2004).

In addition to controlling disease, antimicrobial agents have a critical role in the treatment of a range of injuries such as open wounds, fractures and post-operative incisions. Furthermore, surface infections which lead to sepsis are a major cause of mortality in burns patients and the causative microbes in these cases cover a range of

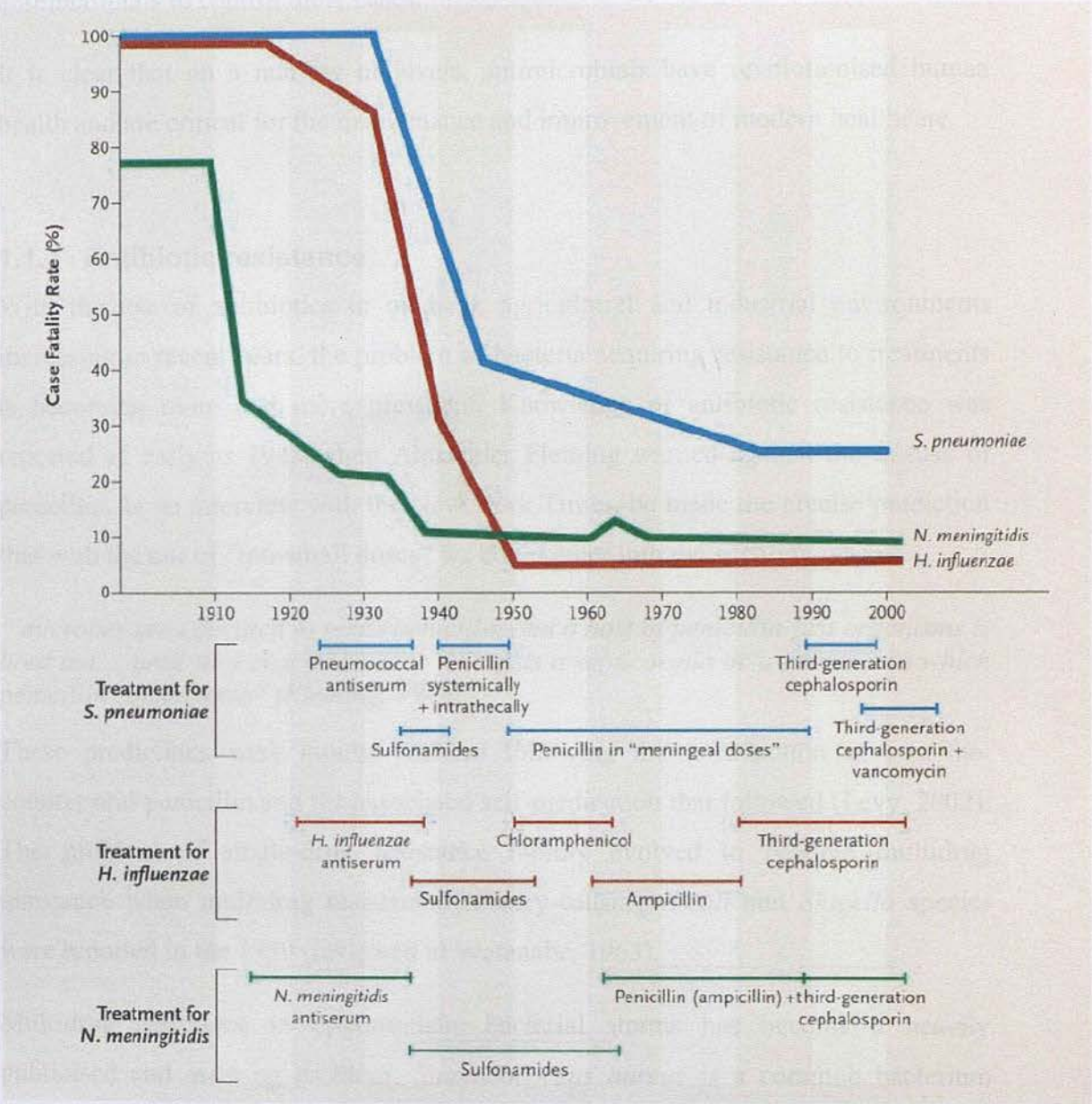


Figure 1.1 Mortality rates associated with bacterial meningitis since from 1910-2000 (taken from Swartz, 2004)
Prior to the introduction of antibiotics, the mortality rates associated with bacterial meningitis caused by *S.pneumoniae*, *H.influenzae* and *N.meningitidis* ranged between 80-100%. The introduction of firstly antisera, then varied antibiotics as treatments for the different strains of disease resulted in the mortality rates dropping to 7-30% by 2000.

bacterial and fungal species (Mahar *et al.*, 2010), underlining the need for effective antimicrobials to control these cases.

It is clear that on a number of levels, antimicrobials have revolutionised human health and are critical for the maintenance and improvement of modern healthcare.

1.1.1 Antibiotic resistance

With the use of antibiotics in medical, agricultural and industrial environments increasing in recent years, the problem of bacteria acquiring resistance to treatments is becoming more and more prevalent. Knowledge of antibiotic resistance was reported as early as 1945 when Alexander Fleming warned against the misuse of penicillin. In an interview with the New York Times, he made the precise prediction that with the use of "too-small doses" we could enter into the situation where:

"microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out.....until they reach someone who gets a septicaemia or a pneumonia which penicillin cannot save" (Fleming, 1946).

These predictions were rapidly realised following the introduction of over-the-counter oral penicillin and the associated self-medication that followed (Levy, 2002). The problem of single-drug resistance rapidly evolved to become multidrug resistance when multidrug resistant dysentery-causing *E.coli* and *Shigella* species were reported in the 1959 (reviewed in Watanabe, 1963).

Multidrug resistance in opportunistic bacterial strains has become a heavily publicised and growing problem. *Staphylococcus aureus* is a common bacterium which is estimated to be carried in the nose and skin of approximately 30% of healthy humans (EARSS Anuual Report, 2008), however it is notorious for causing serious infections in hospitalised patients, which can result in prolonged hospital stays and mortality rates of up to 50% (Cosgrove *et al.*, 2003). Methicillin-resistant *S.aureus* species (MRSA) were first reported in the UK in 1961 (Jevons, 1961), however the incidence of MRSA-related deaths in the UK increased more than 15-fold between 1993 and 2002 (Figure 1.2, UK National Statistics Office), illustrating the growing problem of resistance. Current estimates suggest that up to 50% clinical *S.aureus* isolates in Europe are methicillin-resistant (Figure 1.3, EARSS Annual

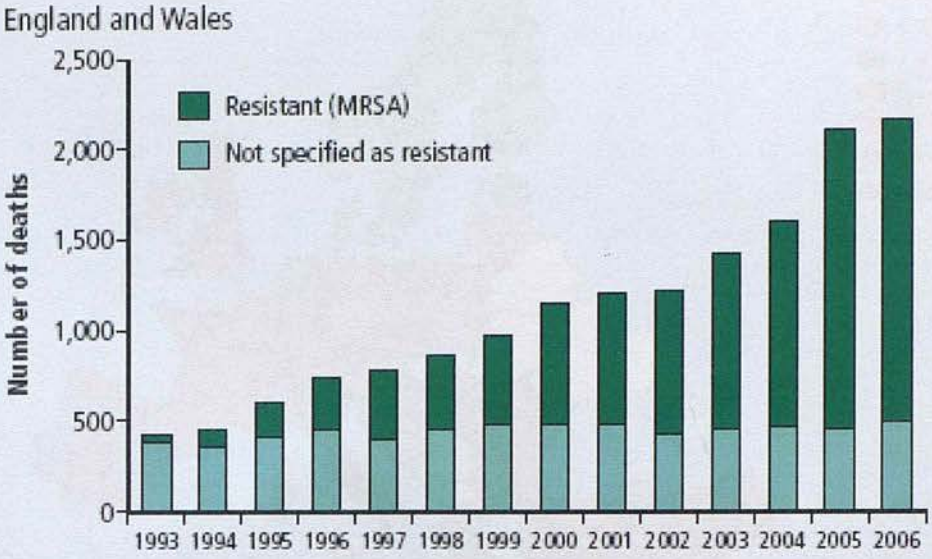


Figure 1.2 Incidence of *MRSA*-related deaths in England and Wales from 1993-2006 (taken from www.statistics.gov.uk)

The incidence of deaths related to MRSA in England and Wales rose steadily from 1993-2006, while deaths from methicillin-sensitive strains remained constant.

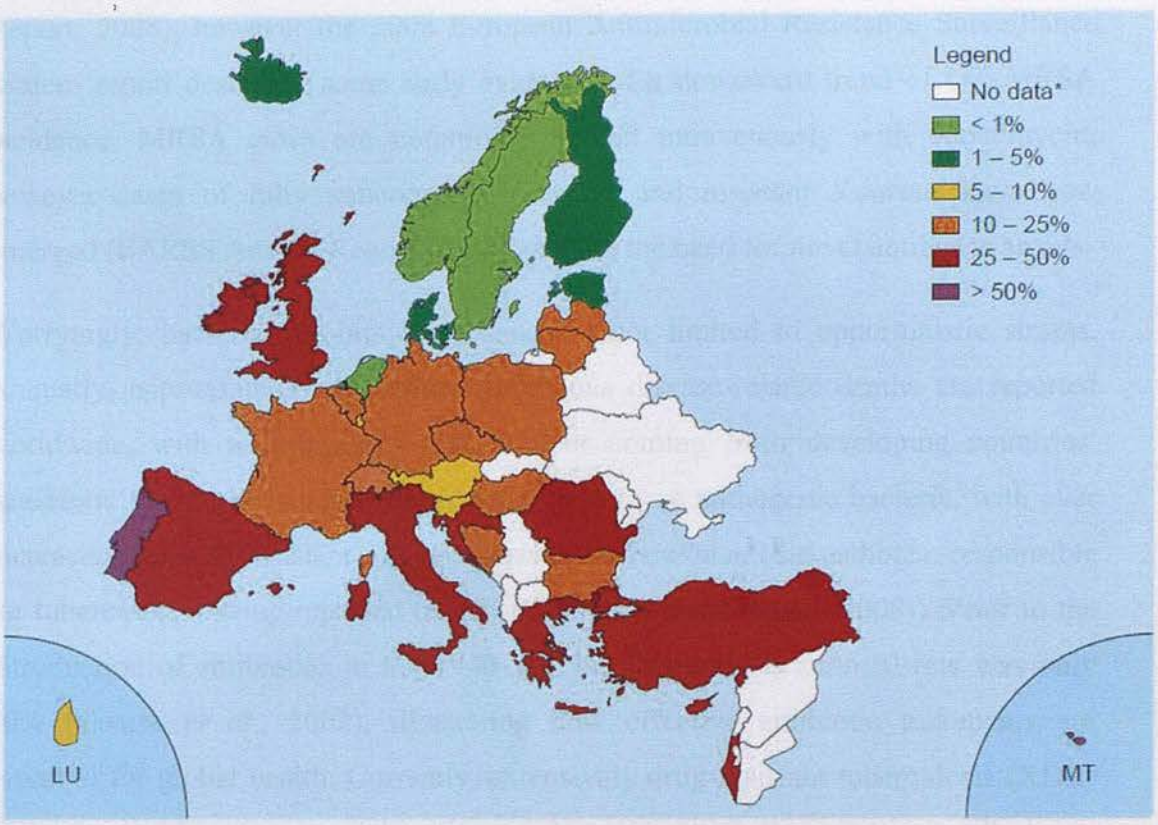


Figure 1.3 Reported levels of oxacillin-resistant *S.aureus* species obtained from clinical isolates across Europe in 2008** (taken from EARSS report, 2008)

*countries labelled in white did not submit data
** oxacillin is similar in structure to methicillin and has replaced methicillin in clinical use

Report, 2008), however the 2008 European Antimicrobial Resistance Surveillance System report described some early evidence of a downward trend of UK MRSA incidence. MRSA cases are commonly treated intravenously with vancomycin, however cases of fully vancomycin sensitive and resistant *S.aureus* have now emerged (EARSS Annual Report, 2008), driving the need for novel antibiotic agents.

Worryingly, bacterial antibiotic resistance is not limited to opportunistic strains. Annually, approximately 15 million infectious disease-related deaths are reported worldwide, with a disproportionate number coming from developing countries. Antibiotic resistance is beginning to emerge in these pathogenic bacteria, with ever increasing cases of resistant *Mycobacterium tuberculinum* (the pathogen responsible for tuberculosis) being reported (reviewed in (Jain and Mondal, 2008)). Prior to the introduction of antibiotics in the 1940-50s, the tuberculosis survival rate was only 50% (Coates *et al.*, 2002), illustrating how effective antibiotic treatments are essential for global health. Currently, extensively drug-resistant tuberculosis (XDR-TB) is treatable using a combination of drugs to which the organism still shows susceptibility (Jassal and Bishai, 2009). The problem of XDR-TB is worsened by the incompatibility of certain antibiotics with drugs used to treat HIV (Koul *et al.*, 2011), reducing the number of drugs available to treat tuberculosis in HIV-positive patients. This, and the emergence of fully resistant tuberculosis may one day render the disease untreatable.

The emergence of multidrug resistant bacteria serves to remind us of how modern healthcare is dependent on effective and reliable antimicrobial agents.

1.1.2 The slowing of novel antibiotic development

In the last three decades, only two new categories of antibiotic have been successfully developed and marketed (Linezolid in 2000 and Daptomycin in 2003) (Coates *et al.*, 2002; Woodworth *et al*, 1992) with all others being modifications of existing drugs. Although the global market for antimicrobial agents in 2008 was estimated to be worth US $24billion, a number of key pharmaceuticals no longer develop new drugs in the field (for example, Roche), and Eli Lilley, the company

which developed vancomycin, currently has no novel antimicrobials in their production pipeline. This slowing in interest in antimicrobials is largely believed to be happening due to the increased financial incentive of producing drugs to treat chronic illnesses which require longer courses of drugs. In addition, the rate at which resistant strains emerge to novel antibiotics shortens the financial lifespan of the drugs, making them a less attractive research area.

The problem of antibiotic resistance is being addressed by implementing a number of measures to slow its progression. For example, reducing the misuse of antibiotics (often the mis-prescription of antibiotics for viral complaints) as well as increasing hygiene and sterilisation protocols in hospitals has led to an apparent containment in the number of reported cases of MRSA in the UK (EARSS Annual Report, 2008). However, these methods will only slow the spread of fully resistant pathogenic bacteria and therefore there is a vital requirement for novel antibiotics. One promising source of novel drugs may come from the study and development of natural antimicrobial peptides of the innate immune system.

1.2 ANTIMICROBIAL PEPTIDES OF THE INNATE IMMUNE SYSTEM

Antimicrobial peptides are diverse and are distributed throughout both the plant and animal kingdoms, where they provide a non-specific "first defence" against invading microbes. They are generally short, positively charged (cationic) peptides which fold to form amphiphilic structures. This optimises their ability to insert specifically into microbial cell walls in order to permeabilise them (Ganz, 2003). Furthermore, antimicrobial peptides possess a broad, non-specific spectrum of antibiotic activity and are microbicidal to a variety of organisms, including both gram positive and negative bacteria, enveloped viruses, fungi and protozoa (Zasloff, 2002). To further this effect, certain peptides have been shown to modulate the adaptive immune response by recruiting and activating key cells to sites of infection (Figure 1.4).

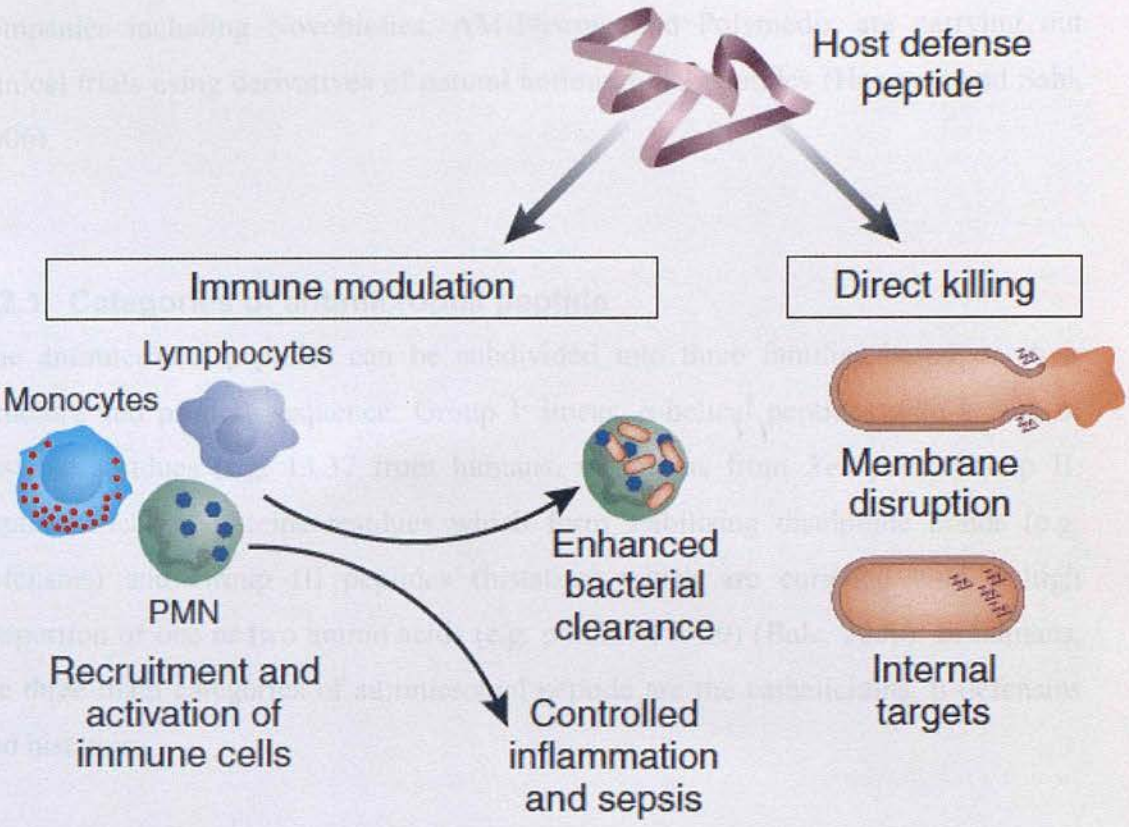


Figure 1.4 Roles of innate host antimicrobial peptides (taken from Hancock and Sahl, 2006)

Host antimicrobial peptides taken on a dual role in fighting microbial attacks. The peptides directly attack microbial membranes while recruiting cells from the adaptive immune system to the site of infection.

Despite the high costs associated with peptide synthesis, the above properties make antimicrobial peptides attractive candidates for novel antibiotic drugs. Accordingly, companies including Novobiotics, AM-Pharma and Polymedix are carrying out clinical trials using derivatives of natural antimicrobial peptides (Hancock and Sahl, 2006).

1.2.1 Categories of antimicrobial peptide

The antimicrobial peptides can be subdivided into three families based on their structure and primary sequence: Group I: linear, α-helical peptides with a lack of cysteine residues (e.g. LL37 from humans, magainins from *Xenopus*); Group II: peptides rich in cysteine residues which form stabilising disulphide bonds (e.g. defensins) and Group III peptides (histatins) which are enriched with a high proportion of one or two amino acids (e.g. porcine PR-39) (Bals, 2000). In humans, the three main categories of antimicrobial peptide are the cathelicidins, β-defensins and histatins.

1.3 THE DEFENSIN FAMILY

The defensins comprise one of the largest, most common groups of antimicrobial peptides and are found in species throughout the animal and plant kingdom, including fungi, arthropods, plants, molluscs, primates, chickens and platypus (Froy and Gurevitz, 2003; Lehrer, 2004; Patil *et al.*, 2004; Patil *et al.*, 2005; Thomma *et al.*, 2002; Whittington *et al.*, 2008; Xiao *et al.*, 2004).

They are small (3.5-4.5kDa in mammals), positively charged (cationic) cysteine-rich peptides, characterised by the presence of even numbers of conserved cysteine residues. These residues form sets of intramolecular disulphide bonds which act to stabilise the peptides' secondary and tertiary structure. In mammals, the defensins can be further sub-divided into α-, β- and θ-defensins according to the spacing and connectivity of their conserved cysteine residues, as well as their overall structure; the α-defensins fold with disulphide bonds forming between cysteines C1:C6, C2:C4 and C3:C5 (Zhang *et al.*, 1992), the β-defensins are stabilised by bonding between

cysteine residues C1:C5, C2:C4 and C3:C6 (Tang *et al.*, 1993) and the cyclic θ-defensins cysteines adopt the conformation C1:C6, C2:C5 and C3:C4 (Tang *et al.*, 1999). These conformations are summarised in Figure 1.5.

The specific categories of defensin have not yet been observed in organisms outside the mammalian family; however defensin-like peptides exist in other species, which often adopt a tertiary structure similar to that of the β-defensins (Figure 1.6). The evolutionary relationship between these defensins and mammalian defensins remains somewhat unclear but one theory argues that they all descended from a common ancestral gene following gene duplication events and subsequent divergence (reviewed in Hughes, 1999). However, the defensins have been subject to very rapid evolution driven by positive selection (reviewed in (Semple *et al.*, 2006)) and share very little primary sequence homology between and within subfamilies and species (their homology lies at the level of secondary and tertiary structure). This has led to proposals that the plant, platypus and insect defensins have arisen via convergent evolution, and share no evolutionary relationship with the rest of the β-defensin group (Froy *et al.*, 2005; Whittington *et al.*, 2008). However, a contradictory report has found that, based on 3D structure, the homology between plant and insect defensins and human β-defensins is greater than that between the human α- and β-defensins. This supports the suggestion in Hughes, 1999 that a single ancestral eukaryotic defensin is likely to have originated prior to the divergence of plants and animals (Thomma *et al.*, 2002).

Additional support for the divergent evolution of the vertebrate α- and β-defensin families was reported in Xiao *et al.*, 2004 where comparative analysis of the chicken, mouse and human genomes suggested that all modern defensins evolved from a single ancestral β-defensin-like gene. This appears to have undergone rapid duplication throughout evolution to give rise to the clusters of defensins seen in current genomes (Xiao *et al.*, 2004).

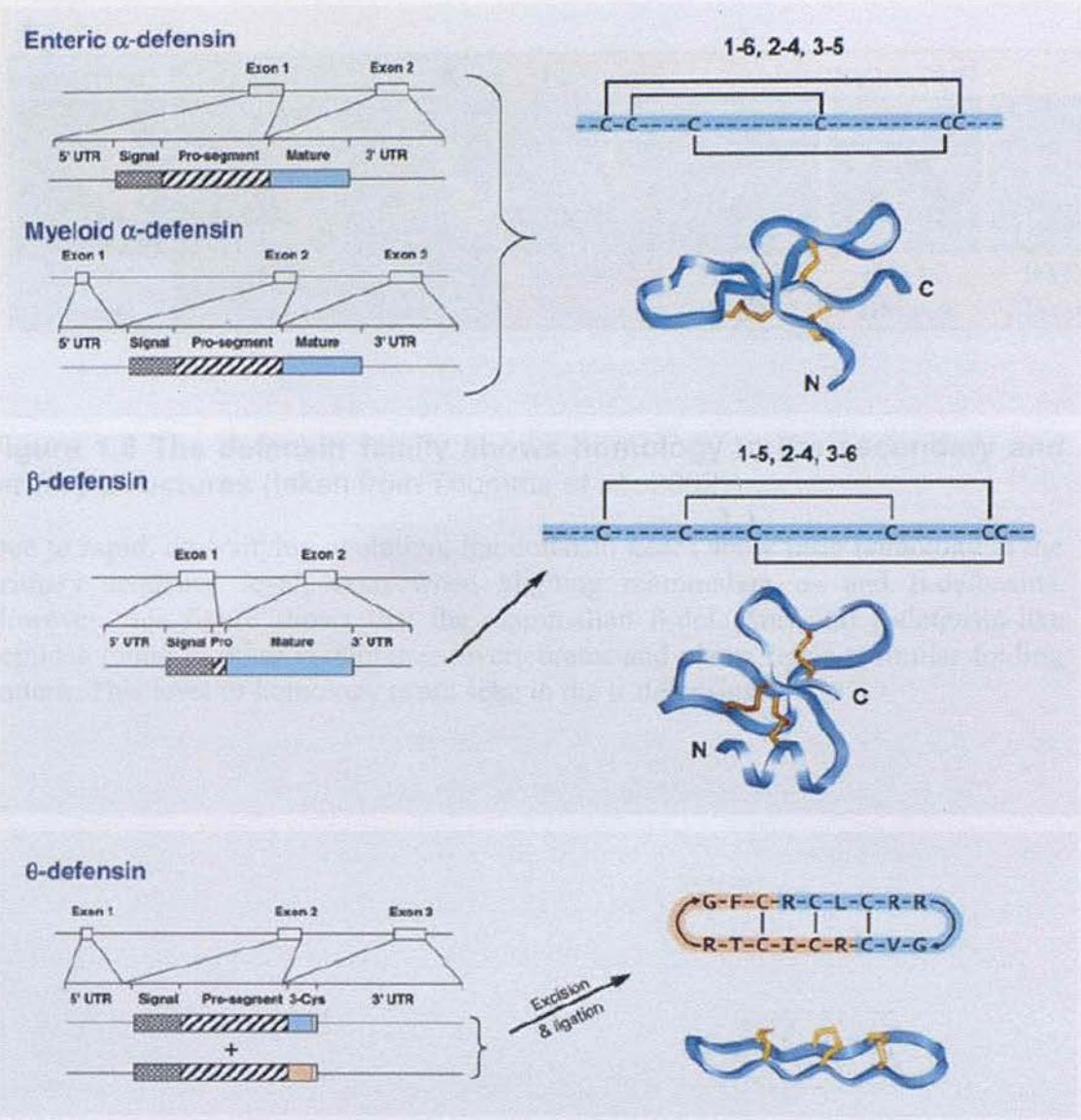


Figure 1.5 The mammalian defensin subfamilies (taken from Selsted and Ouellette, 2005)
The defensin subfamilies are largely categorised according to the spacing and connectivity of their six conserved cysteine residues. As shown above, the α-defensins show a C1:C6, C2:C4, C3:C5 pattern, the β-defensins show a C1:C5, C2:C4, C3:C6 pattern and the circular θ-defensins show C1:C6, C2:C5 and C3:C6 connectivity.

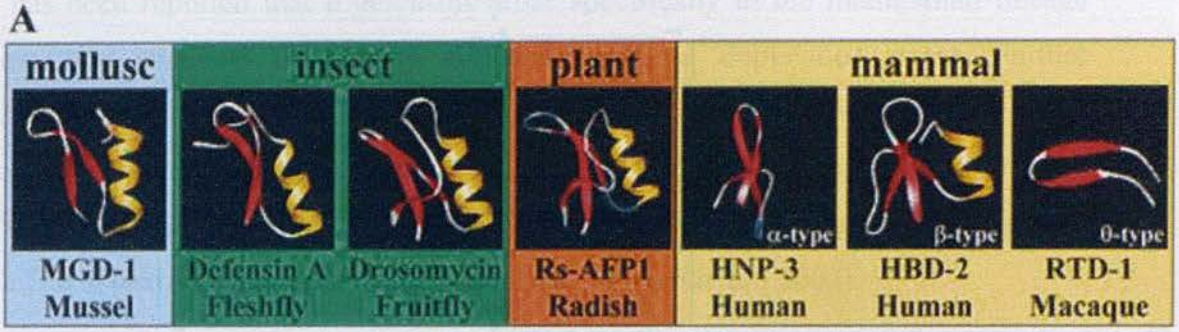


Figure 1.6 The defensin family shows homology in the secondary and tertiary structures (taken from Thomma *et al.*, 2002)

Due to rapid, diversifying evolution, the defensin genes show little homology at the primary sequence level, even when aligning mammalian α- and β-defensins. However, this figure shows that the mammalian β-defensins and β-defensin-like peptides found in other vertebrates, invertebrates and plants retain a similar folding pattern. This level of homology is not seen in the α-defensins.

It has been reported that α-defensins arose specifically in the mammalian lineage from two ancestral β-defensins following a gene duplication event. Further duplications and positive, diversifying selection are thought to have given rise to the modern α-defensin cluster (Patil *et al.*, 2004). Θ-defensins are thought to have originated from the fusion of two ancestral α-defensins, following the divergence of the Old World Monkeys from other hominids (Nguyen *et al.*, 2003).

Regardless of their species, the defensins are best-known for their antimicrobial activity. However, roles for these molecules are beginning to emerge in a variety of processes including the chemotaxis of key adaptive immune response cells, wound repair and inflammation (summarised in Figure 1.7).

1.3.1 The α-defensins

The α-defensins were first discovered as isolates from human and rabbit neutrophil granules (Ganz *et al.*, 1985; Selsted *et al.*, 1985) and were the first members of the defensin family to be identified. The name "human neutrophil peptide" (HNP) was coined and it was found that a mixture of HNP1, HNP2 and HNP3 was able to kill both gram positive and gram negative bacteria (*S.aureus*, *P.aeruginosa* and *E.coli*).

α-defensins are encoded by genes comprising three exons, initially synthesised as prepropeptides (Lehrer *et al.*, 1993) and a cluster containing HNP1-4 has been mapped to human chromosome 8p23.1 (Sparkes *et al.*, 1989).

Polymorphonuclear leukocytes (PMNs) synthesise, process and package HNP1-4 into their azurophil granules (Valore and Ganz, 1992). These myeloid defensins are synthesised as inactive precursors and metabolic labelling studies have shown that these preprodefensins are processed to form mature peptides via two intermediates: a 75 amino acid, cytoplasmic preprodefensin, (containing a signal sequence) and a 56 amino acid, inactive prodefensin.

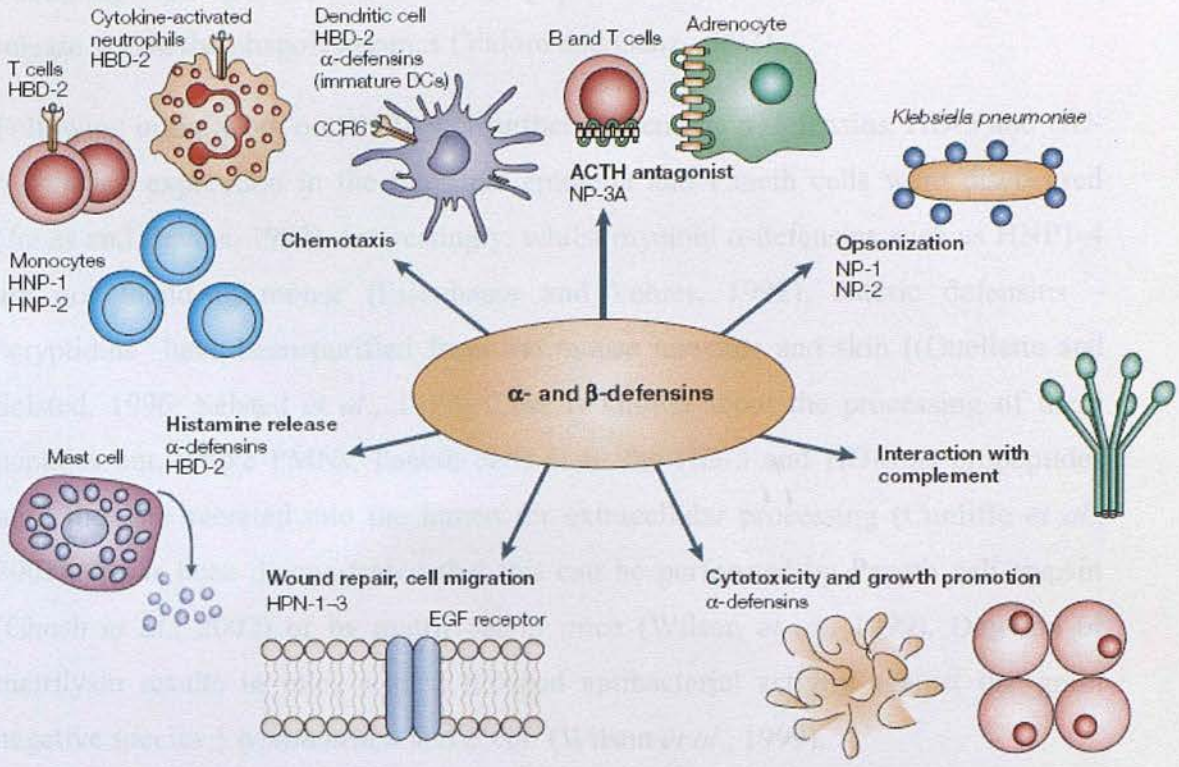


Figure 1.7 The α- and β- defensins have varied, diverse roles in host defence (taken from Lehrer, 2004)

The defensins are best known for their antimicrobial activity however they also have a role in a number of additional defence mechanisms including wound repair, chemotaxis, opsonisation and inflammation.

Following cleavage, the active mature peptide is stored in granules ready for its release within the phagolysosomes (Valore and Ganz, 1992).

Following initial work on HNP1-4, a further two enteric α-defensins, HD-5 and HD-6 showing expression in the intestinal epithelia and Paneth cells were discovered (Jones and Bevins, 1992). Interestingly, whilst myeloid α-defensins such as HNP1-4 are not found in mouse (Eisenhauer and Lehrer, 1992), enteric defensins – "cryptidins" have been purified from the mouse intestine and skin ((Ouellette and Selsted, 1996; Selsted *et al.*, 1992). Less is known about the processing of these peptides but, unlike PMNs, Paneth cells store the HD-5 and HD-6 as propeptides until they are secreted into the lumen for extracellular processing (Cunliffe *et al.*, 2001). It has been demonstrated that this can be performed by Paneth cell trypsin (Ghosh *et al.*, 2002) or by matrilysin in mice (Wilson *et al.*, 1999). Deletion of matrilysin results in mice with a reduced antibacterial activity against the gram negative species *S.typhimurium* and *E.coli* (Wilson *et al.*, 1999).

1.3.2 α-defensin structure and function

As described previously, the α-defensins form a tertiary structure stabilised by disulphide bonds formed between cysteine residues C1:C6, C2:C4 and C3:C5 (Zhang *et al.*, 1992). Analysis of the crystal structure of HNP-3 revealed that its three internal disulphide bonds stabilise a dimeric tertiary conformation consisting mainly of a three-strand, antiparallel β-sheet (Hill *et al.*, 1991). X-ray-based structural analysis of HD-5 and HD-6 revealed that these peptides also form dimers in solution and that the tertiary structures of HNP3 and 4 are highly similar to HD-5 and HD-6 (Szyk *et al.*, 2006)(Figure 1.8).

Since the discovery of the α-defensins, much work has been carried out to further characterise the range of their antimicrobial activity. Strikingly, the activity of the individual defensins is variable and not limited to bacterial killing – HNP1 can directly inactivate herpes simplex virus (HSV) 1 and 2, cytomegalovirus and influenza by incubation alone (Daher *et al.*, 1986), as well as showing activity against the fungus *Candida albicans* (Lehrer *et al.*, 1988). In contrast, HNP2 and

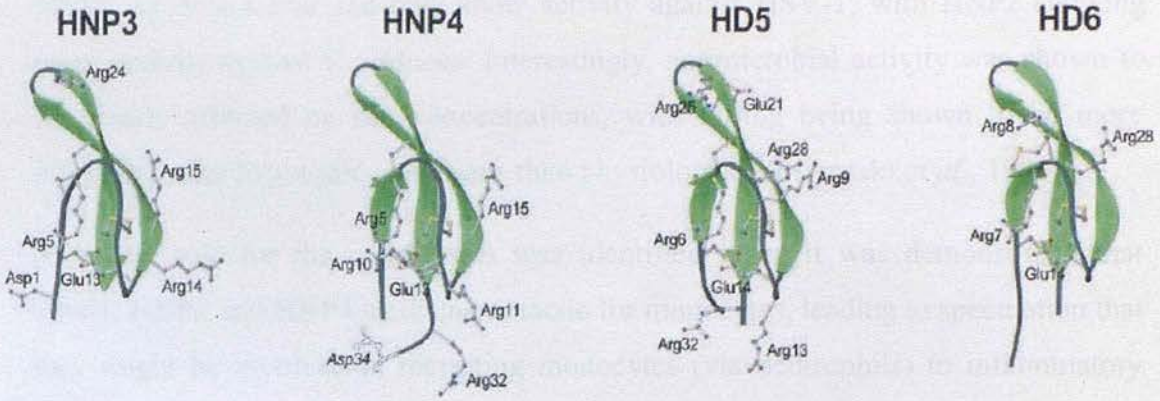


Figure 1.8 The tertiary structure of the α-defensins (taken from Szyk *et al.*, 2006)

Analysis of these four human α-defensins revealed a conserved tertiary structure, comprising a triple strand anti-parallel β-sheet.

HNP3 are less active and only show activity against HSV-1, with HNP2 showing some activity against *C.albicans*. Interestingly, antimicrobial activity was shown to be greatly affected by salt concentrations, with killing being shown to be more effective under hypotonic conditions than physiological (Miyasaki *et al.*, 1990).

A further role for the α-defensins was identified when it was demonstrated that HNP1, HNP2 and HNP4 were chemotactic for monocytes, leading to speculation that they might be involved in recruiting monocytes (via neutrophils) to inflammatory sites ((Territo *et al.*, 1989). This is supported by the finding that human neutrophil defensins selectively induce $CD4^+$ and $CD8^+$ naive T cells and are chemoattractive for immature dendritic cells. This activity is abrogated by treatment with pertussis toxin, suggesting that a G-coupled receptor is involved (Yang *et al.*, 2000). Finally, HNPs have also been shown to strongly bind C1q following activation of the complement system (Panyutich *et al.*, 1994).

1.3.3 The θ-defensins

The θ-defensins represent the newest members of the defensin family and were identified in 1999 from the leukocytes of Rhesus macaques (Tang *et al.*, 1999). They are 18-residue peptides characterised by a distinct cyclic structure. Rhesus θ-defensin 1 (RTD-1), the first reported θ-defensin, is the product of the posttranslational "head-to-tail" ligation of two α-defensin-related nonapeptides, RTD1a and RTD1b (Tang *et al.*, 1999). As with the α- and β-defensins, the structure is stabilised by intramolecular disulphide bonds (Figure 1.9).

RTD-1 is antimicrobial towards both gram positive and gram negative bacteria, and fungi including *C.albicans*. This activity is salt-insensitive, a trait thought to be conferred by the peptide's cyclic nature. It has been speculated that this cyclic structure makes the peptide more resistant to inflammation-recruited proteases and exopeptidases (Tang *et al.*, 1999).

Two further θ-defensins, RTD-2 and 3 have also been purified from the bone marrow of rhesus monkeys (Leonova *et al.*, 2001). These peptides are homologous to RTD-1 and are predicted splice-products of RTD1a and 1b (Tran *et al.*, 2002). Like RTD-1,

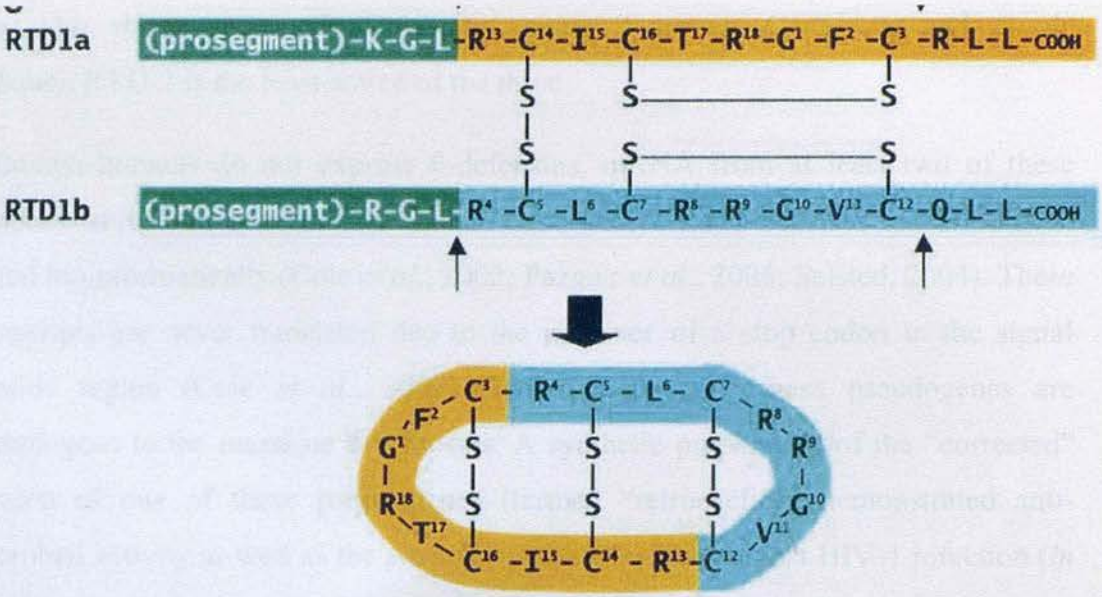


Figure 1.9 The θ-defensins are formed by a head-to-tail ligation of two α-defensin-like precursors (taken from Tang *et al.*, 1999)

The θ-defensins are formed via the posttranslational head-to-tail ligation of two nonapeptide
precursors, RTD1a and RTD1b. This occurs via an unknown mechanism.

they also show antimicrobial activity against *S.aureus*, *C.albicans* and *E.coli*, although RTD-2 is the least active of the three.

Although humans do not express θ-defensins, mRNA from at least two of these peptides have been isolated from human bone marrow, and a further four have been found bioinformatically (Cole *et al.*, 2002; Pazgier *et al.*, 2006; Selsted, 2004). These transcripts are never translated due to the presence of a stop codon in the signal peptide region (Cole *et al.*, 2002). The sequences of these pseudogenes are homologous to the macaque θ-defensins. A synthetic preparation of the "corrected" version of one of these pseudogenes (termed "retrocyclin") demonstrated anti-microbial activity as well as the protection of CD4+ cells against HIV-1 infection (*in vitro*) by inhibiting proviral DNA formation. This interesting finding has led to work investigating the possibility for "reawakening" human θ-defensin pseudogenes as potential therapeutics (Venkataraman *et al.*, 2009). Although the mechanism by which the nonapeptide precursors of the θ-defensins are ligated is unknown, it has recently been shown that human cells are capable of carrying out this process *in vivo*. By utilising aminoglycoside treatment to reduce the integrity of translation (thereby bypassing the pseudogene stop codon), functional θ-defensin peptides have been produced in human cells (Venkataraman at al., 2009). Since this activity protected the cells from HIV-1 infection, this θ-defensin research presents interesting opportunities for the field of HIV therapeutics and prophylactics.

1.3.4 The β-defensins

The first β-defensin was isolated from bovine tracheal epithelial cells ("tracheal antimicrobial peptide, TAP"), where it was noted as being similar to, but distinct from the α-defensins (Diamond *et al.*, 1991). This was followed by the discovery of thirteen structurally homologous peptides from bovine blood neutrophils (BNBD1-13) (Selsted *et al.*, 1993) whereupon this new family were reclassified as the β-defensins. Further work demonstrated that the β-defensins showed different cysteine spacing and connectivity patterns to the α-defensins (Figure 1.5) (Tang and Selsted, 1993), with a characteristic C1:C5, C2:C4 and C3:C6 binding pattern. The β-defensin family in humans is located in the same locus as the α-defensins at 8p23.1.

1.3.5 The structure and interactions of β-defensins

As with the α-defensins, the β-defensin genes encode prepropeptides consisting of a signal sequence, pro-sequence and six-cysteine mature peptide (Patil *et al.*, 2005). Generally, in mammals the peptides are encoded over two exons, with the signal and pro-sequence encoded on the first exon and the mature peptide on the second.

The mature β-defensins fold to form an amphipathic structure comprising a triple-stranded antiparallel β-sheet, stabilised by intramolecular disulphide bonds, in a similar way to the α-defensins. However, unique to the β-defensin structure is an N-terminal α-helix, which may be important for the peptide's chemotactic properties (J Lubkowski, personal communication).

1.4 HUMAN AND MURINE β-DEFENSINS

The first mammalian β-defensin to be documented was TAP (see previous section), which was rapidly followed by lingual antimicrobial peptide (LAP), which was isolated from bovine tongues (Schonwetter *et al.*, 1995). Since then the isolation and characterisation of the mammalian β-defensins has blossomed. In this section, the well-characterised peptides in human and mouse will be described and the association of variant β-defensin expression and disease will be discussed.

1.4.1 Human β-defensins

Since their initial discovery, four human β-defensins have been characterised in detail, with more than 28 others being reported using bioinformatical searches of the human genome (Schutte *et al.*, 2002).

Human β-defensin 1 (HBD1) was originally isolated from human blood filtrates, where it was noted that it shared homology to the previously described bovine and chicken β-defensins (Bensch *et al.*, 1995). Constitutive HBD1 expression has been detected in normal epithelia from the trachea, bronchi, airways, mammary gland, salivary gland, testis, thymus, small intestine and skin (Fulton *et al.*, 1997; McCray

and Bentley, 1997; Zhao *et al.*, 1996), with particularly high concentrations being detected in the kidney, loops of Henle, reproductive tract and urine (Valore *et al.*, 1998).

As with the α-defensins, recombinant HBD1 showed salt-sensitive bactericidal activity against *E.coli* (Valore *et al.*, 1998), however, the expression of HBD1 is not up-regulated in response to infection. A recent report has suggested that HBD1 becomes a more potent antimicrobial under reducing (anaerobic) conditions. It was shown that following the reduction of the peptide's disulphide bonds, the peptide acquired effective antimicrobial activity against gram positive gut commensals (Schroeder *et al.*, 2011).

Human β-defensin 2 (HBD2) was isolated following the observation that patients with psoriasis suffer from fewer skin infections than expected, and it was hypothesised that psoriatic skin lesions might produce antimicrobial peptides. HBD2 was purified from these lesions and found to be antimicrobial to *P.aeruginosa* and *C.albicans*, but was ineffective against gram positive strains (Harder *et al.*, 1997). However, this activity is salt-sensitive (Bals *et al.*, 1998).

Unlike *HBD1*, *HBD2* is up-regulated in response to TNFα and bacteria, and was the first defensin found to be up-regulated in this manner. In addition, constitutive expression of HBD2 was detected in the lung and trachea, with lower levels being found in the kidney, uterus and salivary gland (Harder *et al.*, 1997). More recently, it has been shown that Th17 cells (a subset of $CD4^+$ T cells) also induce *HBD2* via the cytokine IL-22 (Liang *et al.*, 2006).

Human β-defensin 3 (HBD3) was initially identified as an anti-gram positive factor from psoriatic scales cloned from keratinocytes (Harder *et al.*, 2001). This followed the discovery that psoriatic lesion-derived HBD2 had no effect on gram positive bacteria. Impressively, HBD3 demonstrates a salt-insensitive spectrum of antimicrobial activity against MRSA, vancomycin-resistant *E.faecium* and the notoriously hardy *B. cepacia* (Garcia *et al.*, 2001a; Harder *et al.*, 2001). Moreover, structural analysis of *S.aureus* treated with HBD3 revealed signs of cell wall perforation, demonstrating a possible mechanism of action for the peptide (Harder *et*

al., 2001). The reported action of HBD3 against *B.cepacia* is particularly significant in the pathology of cystic fibrosis (CF), where no effective antibiotic treatment is available for CF sufferers with acute *B.cepacia* infections. This organism is notoriously resistant to both antimicrobial peptides and antibiotics and has even been reported to have utilised penicillin as a carbon source (Beckman and Lessie, 1979).

Expression of HBD3 is observed in the epithelia of multiple organs, including the heart, skeletal muscle, placenta, skin, tonsils, oesophagus, testis trachea and foetal thymus (Jia *et al.*, 2001). In addition, this expression is increased following stimulation with IFN-γ , TNFα, IL-1β and contact with bacteria (Garcia *et al.*, 2001a; Harder *et al.*, 2001; Jia *et al.*, 2001).

Human β-defensin 4 (HBD4) was first detected following genomic searches (mainly BLAST-based)(Garcia *et al.*, 2001b). The distribution of its transcripts was widespread and the highest recorded expression was found in the testis. Lower levels of expression were also found in the uterus, neutrophils, thyroid gland, lung and kidney, but no detectable expression was reported elsewhere (Garcia *et al.*, 2001b). As with HBD2 and HBD3, HBD4 was found to be induced upon treatment with heat-killed *P.aeruginosa* and *S.pneumoniae*, however single immune components such as IL-1α, IL-6, IFN-γ or TNFα had no effect on expression.

As with HBD1 and HBD2, HBD4 showed activity against gram negative bacteria and weak activity against gram positive, but this activity was salt-sensitive.

HBD5 and *HBD6* were discovered following a "mining" approach on the human genome, and epididymis-specific expression was reported for these peptides (Yamaguchi *et al.*, 2002). Following the discovery of *HBD5* and *HBD6*, more complex genomic searches utilising hidden Markov models in combination with BLAST searches identified a further 28 new human β-defensins, along with 43 mouse peptides (Schutte *et al.*, 2002). Preliminary expression studies completed at the time suggested that many of these sequences were transcribed, although a number of these were pseudogenes and included premature stop codons.

1.4.2 Murine β-defensins

As with the human β-defensins, more murine β-defensins have been reported than have been functionally analysed. As of February 2011, 49 murine defensins were described on the Ensembl database, however only three key genes will be discussed in this section.

The first murine β-defensin to be detected was *Defb1*, a homologue of *HBD1* showing expression at high levels in the kidney, with lower expression in the brain, heart, lung, uterus, spleen, muscle, stomach and small intestine (Huttner, 1997). Like *HBD1*, the gene was found to map to chromosome 8 with the α-defensin cluster and the genomic organisation of the gene (two exons, separated by a single long intron) was similar to that of *HBD1* (Bals *et al.*, 1998; Morrison *et al.*, 1998).

The activity of Defb1 was again, similar to HBD1 with salt-sensitive activity against *P.aeruginosa*, but also displayed bactericidal effects on the gram positive bacteria *E.coli* and *S.aureus* (Morrison *et al.*, 1998).

Defb1$^{-/-}$ mice were not hampered in the clearance of *S.aureus* from their airways, but were found to excrete a significantly higher level of *Staphylococcus* species in the bladder, suggesting a role for Defb1 in the resistance of urinary tract infections (Morrison *et al.*, 2002). Further characterisation of this model showed that mutant mice also exhibited a delayed clearance of *H.influenzae* from the lung (Moser *et al.*, 2002).

Murine β-defensin 2 (*Defb2*) was reported and found to be expressed in the lungs, trachea, skin, uterus and kidney (Morrison *et al.*, 1999) and its expression was found to be inducible in response to LPS. This induction following an inflammatory stimulus suggests that it may contribute to host airway defence. In addition, it has also been shown to bind the chemokine receptor, CCR6 to participate in the chemoattraction of bone marrow-derived immature dendritic cells (Biragyn *et al.*, 2001).

Defb3 was initially identified as being a homologue of *HBD2*. Transcripts were detected at low levels in the epithelia of the intestine, airways, bowel, liver and lung and were found to be up-regulated in response to *P.aeruginosa* inhalation (Bals *et*

al., 1999). The peptide was shown to be antimicrobial towards both *P.aeruginosa* and *E.coli* in a salt-sensitive manner (Bals *et al.*, 1999).

The synteny of the human β-defensin cluster at 8p23.1and the related murine orthologues are depicted in Figure 1.10.

1.4.3 The association of β-defensins with human disease

The β-defensin clusters lies at chromosome band 8p23.1, a site which is known to be a frequent site of chromosome rearrangements. In 2003, it was found that this region is also subject to copy number variation, with most normal individuals carrying between 2 and 7 copies (Hollox *et al.*, 2003). A number of studies have since investigated the effects of this variation and several key associations with high and low variation and disease have been made.

Crohn's disease is a chronic intestinal disorder, characterised by gut inflammation. An individual's first occurrence of Crohn's disease often arises following a bacterial infection (Stallmach and Carstens, 2002), and it has been suggested that a defect in β-defensin induction may be the reason gut inflammation occurs (Wehkamp *et al.*, 2005). This model is also supported by the finding that patients with an *HBD2* copy number of less than 4 are more prone to Crohn's disease (Fellermann *et al.*, 2006). However, there is a level of controversy in the field, with conflicting reports suggesting that increased copy number of HBD2 associates with the disease (Bentley *et al.*, 2010).

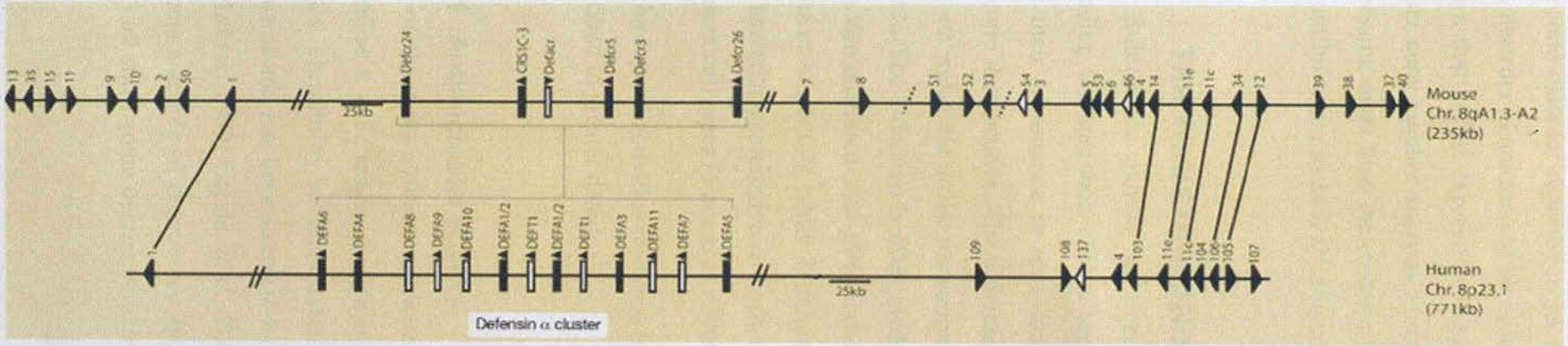


Figure 1.10 Conserved synteny between the primary human and mouse defensin clusters (taken from Whittington *et al.*, 2008)

The human defensin cluster are 8p23.1 shows conserved synteny with murine chromosome 8qA1.3-A2. Here, both clusters show a conserved α-defensin cluster flanked on both sides by β-defensin genes, many of which are orthologous.

A second disorder reported to have β-defensin aetiology is psoriasis. Psoriasis is a skin disorder characterised by redness, scaling and elevated "plaques". It has been shown that *HBD2* expression is high in psoriatic lesions (Harder *et al.*, 2007), but further copy number analysis of the β-defensin cluster has revealed that individuals possessing more than the modal copy number of 4 cluster copies show an increased susceptibility to psoriasis (Hollox and Armour, 2008).

1.5 THE INDUCTION AND FUNCTION OF THE β-DEFENSINS

The β-defensins exhibit an impressively diverse array of biological activities including antimicrobial effects and immunomodulation. These activities highlight both their importance *in vivo* and their potential for development as topical and systemic therapeutics. The β-defensins are unique within the defensin family in that they are inducible at the transcriptional level in response to a number of microbial stimuli. The induction is often mediated by the Toll-like receptors (TLRs) and it has been demonstrated that LPS activation of TLR2 leads to an induction of *HBD2* (Hertz *et al.*, 2003), while TLR3 induction following treatment with poly I:C, LPS and bacterial peptidoglycan leads to the activation of *HBD1*, *HBD2* and *HBD3* in different cell types (Vora *et al.*, 2004; Schaefer *et al.*, 2005).

The biological activities resulting from the induction of the β-defensins will now be described in greater detail.

1.5.1 Antimicrobial activity

One of the most striking activities of the β-defensins is their collective ability to neutralise a wide range of microbes ranging from fungi and bacteria to enveloped viruses. This ability has been tested (*in vitro*) in the majority of reported, purified β-defensins and has been found to be most effective in low salt concentrations (Ganz and Lehrer, 1999; Garcia *et al.*, 2001b; Selsted *et al.*, 1993). Interestingly, in some respects a degree of degeneracy appears to exist amongst the β-defensins; HBD1, 2 and 3 all show some degree of activity against gram negative bacteria (Garcia *et al.*,

2001a; Harder *et al.*, 1997; Valore *et al.*, 1998), however potent activity against gram positive species seems to be most obvious in HBD3, and is absent in HBD2.

The antimicrobial activity of individual β-defensins is determined by a number of characteristics including their overall positive charge and hydrophobicity (a measure of the molecule's ability to insert into microbial membranes). As described earlier, all β-defensins comprise a set of three antiparallel beta-sheets (stabilised by internal disulphide bonds) and an alpha-helix at the N-terminus. Therefore, the individuality and specificities of the β-defensins has been suggested to originate from the unique and variable arrangements of the non-conserved residues (Sahl *et al.*, 2005; Semple *et al.*, 2003). However, a conclusive and comprehensive explanation for this variation has not yet been published.

The activity of the majority of the mammalian β-defensins has been reported to be salt-sensitive. This has sparked debate in the literature as to whether this trait might play a part in the pathogenesis of cystic fibrosis. Cystic fibrosis is the most common lethal genetic disease in Caucasian populations. It is caused by mutations in the cystic fibrosis transmembrane receptor (CFTR) and results in dysfunctional ion transport across epithelial membranes. The disease affects many systems, but the most severe effects are seen in the lung, where the production of thick, mucus allows for chronic bacterial colonisations and repeated acute infections (Parmar and Nasser, 2005). There are a number of theories which attempt to explain this susceptibility to infection. One proposal suggests that cystic fibrosis epithelia contain elevated sodium chloride concentrations, rendering HBD1 inactive (Goldman *et al.*, 1997). However, subsequent studies have failed to find evidence suggesting an elevation of salt levels in cystic fibrosis airways (Jayaraman *et al.*, 2001).

1.5.2 Proposed mechanisms of antimicrobial activity

There has been much speculation and study into the mechanism by which β-defensins and other antimicrobial peptides function, and to date, there is not yet a conclusive answer. While it is not known how the defensins traverse the cell walls of bacteria, the root of their antimicrobial action is thought to lie at the cell membrane

level. Prokaryotic cell membranes are highly negatively charged and therefore will electrostatically interact with the positively charged defensins. Since the outer leaflets of eukaryotic cell membranes hold little or no charge, they cannot be targeted by the defensins, thus making microbial membranes a unique target for attacking host defensin peptides (Matsuzaki, 1999)(Figure 1.11b).

Initial studies investigated the interaction between the rabbit alveolar macrophage proteins MCP1 and MCP2 with *P.aeruginosa*. It was found that both proteins bound LPS, suggesting that this provides an "anchor" for the peptides to bind the bacteria. The outer membranes were also found to be permeabilised by the peptide and were more efficiently phagocytosed by macrophages (Sawyer *et al.*, 1988). This primary work implied that antimicrobial peptides might function by attacking the cell walls of microbes to permeabilise them and further indicates that the initial attraction between the target cell and peptide is electrostatic. This was supported by later work on indolicidin (a cationic bovine neutrophil peptide), which showed a high affinity for LPS and produced "discrete channels" in *E.coli* cell walls that led to the disruption of the cytoplasmic membrane (Falla *et al.*, 1996).

This early work, along with the molecular structure of β-defensins has led to it being accepted that defensins are membrane-attacking, pore-forming agents (Hancock and Lehrer, 1998). This is also concurrent with the prokaryotic specificity of the β-defensins. The presence of negatively charged bacterial cell wall structures such as LPS (gram negative) and teichoic acid (gram positive) provides a stark contrast to the overall neutral charges of eukaryotic cell membrane's cholesterol inserts and therefore provide unique microbial targets for the β-defensins to attack.

Three key models have been proposed to describe the mechanism and structure of the pores formed in bacterial cell walls – the "barrel-stave" model, the "toroidal"/"worm-hole" model and the "carpet" model (Brogden, 2005; Ganz and Lehrer, 1999, reviewed in Wiesner and Vilcinskas, 2010).

The barrel-stave model (Figure 1.11a) suggests that peptide helices form a "bundle" spanning the membrane, with hydrophilic residues pointing inwards to a central pore.

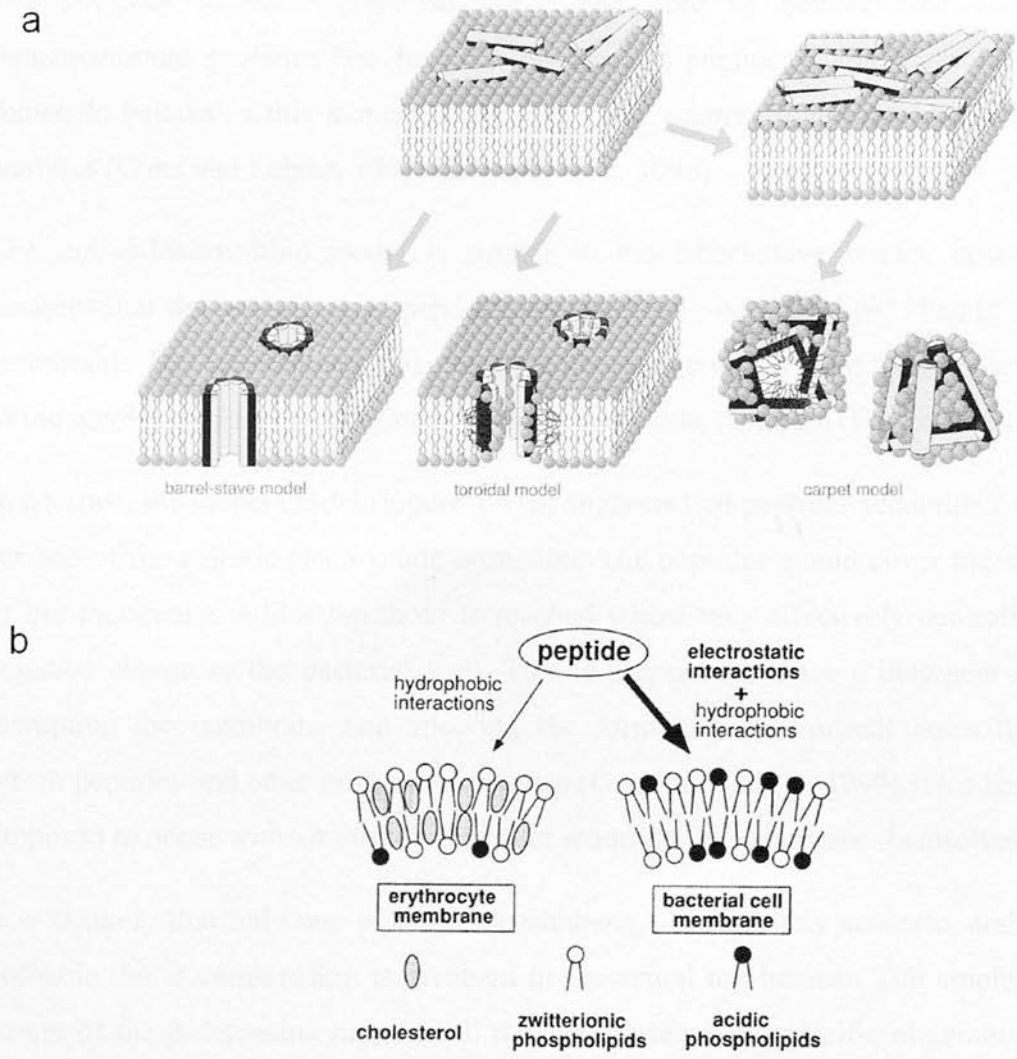


Figure 1.11 Proposed mechanisms of action of antimicrobial peptides

(a) The three proposed mechanisms of antimicrobial action. All three mechanisms rely on the antimicrobial peptides aligning with the bacterial membrane and compromising its integrity. The barrel-stave and toroidal models propose that peptides aggregate at membrane surfaces and insert perpendicularly into the membrane. The barrel-stave model proposes that hydrophobic residues align with the lipid cores and the hydrophilic residues with the interior of the pore. Instead, the toroidal model proposes that the membrane lipids distort so only the lipid cores interact with the antimicrobial peptide. The carpet model proposes that peptides aggregate at the membrane surface, neutralising charge and acting as a detergent. *Figure taken from Wiesner and Vilcinnskas, 2010.*

(b) Due to the zwitterionic nature of eukaryotic membranes, the outer leaflets carry little net charge, leading to hydrophobic interactions with antimicrobial peptides and no membrane breech. However, due to the highly charged acidic nature of prokaryotic membranes, electrostatic interactions between their negative charge and the antimicrobial peptides positive charge allow for membrane infiltration to commence. *Figure taken from Matsuzaki, 1999.*

The peptides would oligomerise across the pore to behave similarly to a transmembrane protein. The fungal antimicrobial peptide, alamethicin has been shown to behave in this manner, following x-ray scattering and neutron scattering analyses (Ganz and Lehrer, 1999; Oren and Shai, 1998).

The toroidal/worm-hole model is similar to the barrel-stave model, however it assumes that the antimicrobial peptides associate only with the lipid "heads" of the membrane. To accommodate this, the lipid membrane would bend to coat the inside of the whole, producing a continuous "worm-hole"-like structure (Figure 1.11a).

In contrast, the carpet model (Figure 1.11a) suggests that peptides accumulate on the surface of the cell via electrostatic attraction. The peptides would cover the surface of the membrane until a threshold is reached where they effectively neutralise the negative charge of the bacterial wall. This is proposed to have a detergent effect, disrupting the membrane and allowing the formation of transient holes through which peptides and other molecules can pass (Ganz and Lehrer, 1999). This has been proposed to occur without the peptides ever spanning the membrane themselves.

It is unlikely that only one of these mechanisms is the entirely accurate, and more probable that a combination is involved in the actual mechanism. The amphipathic nature of the β-defensins supports all models, however the specific oligomerisation necessary for the barrel-stave mechanism to work has not yet been demonstrated in β-defensins (Pazgier *et al.*, 2006). It is also likely that the true mechanism is more complicated than either of the proposed models. An elegant piece of work demonstrated this when model vesicular membranes were synthesised from both random lipid mixes and mixes based on the *E.coli* membrane. Upon treating with rabbit neutrophil defensins, it was shown that the *E.coli* membrane was specifically attacked, causing fast leakage via "large, transient membrane lesions" (Hristova *et al.*, 1997). The presence of transient lesions supports the carpet model, but the specificity of the response implies that other factors are involved.

The mechanism by which this membrane breach causes cell death is also unknown and an added complexity of these studies is the difficulty involved with separating mechanisms of bacterial killing from artefacts of cell death. For example, it has been

shown that Defb118 can kill *E.coli in vitro* following a fifteen minute incubation. However, microscopic analysis of the bacterial cell walls showed cellular damage occurring up to two hours after cell death (Yenugu *et al.*, 2004).

It has also been suggested that following a cell membrane breach, antimicrobial peptides are able to enter the cell to disrupt metabolic processes (Brogden, 2005; Hancock, 1997).

1.5.3 Suitability of the β-defensins as novel therapeutics

The potent antimicrobial activity of the β-defensins makes them good candidates for development into novel antibiotics and antifungals. Clinical trials are currently ongoing to investigate the suitability of derivatives of the peptides for treatment of a number of diseases. It is therefore prudent to consider the mechanisms of resistance which may arise in response to treatment with therapeutic β-defensins.

The resistance mechanisms that microbes already employ and might develop in the future are of extreme importance when considering the use of β-defensins as therapeutics. In particular, the potential for resistance must be understood to guard against cross-resistance between synthetic antimicrobial therapeutics and innate, natural defences. A recurring theme in bacterial resistance mechanisms is the changing of the charge on the bacterial cell wall.

S.aureus is able to tolerate relatively high concentrations of some β-defensins, and HBD2 in particular has no reported activity against this organism. In 1999, a study was undertaken to identify key genes responsible for this insensitivity. A number of mutagenised strains of *S.aureus* showing increased sensitivity to the antimicrobial peptide gallidermin were obtained. It was found that a proportion of these mutants exhibited an altered teichoic acid structure which lacked D-alanine residues. This increased the overall negative charge of the cells, allowing them to bind more positively charged peptides. The sensitivity of these cells extended to HNP1, 2 and 3 (Peschel *et al.*, 1999). This study implies that altering the surface structures and charge of bacterial cells will have a direct effect on their resistance to antimicrobial peptides.

A further gene to have been implicated in bacterial resistance to β-defensins is the virulence factor MprF (Peschel *et al.*, 2001). In this study, it was found that *mprF* mutants demonstrated attenuated virulence *in vivo*, and that these mutants were unable to modify a key inner membrane component phosphatidylglycerol with L-lysine. This normally results in a reduction of net negative charge in the membrane and serves to repel positively charged peptides (Staubitz *et al.*, 2004).

1.5.4 Chemoattractant properties

Chemotaxis is the process by which a cell's movement is defined by an extracellular chemical gradient. It was first observed in an immunological setting when leukocytes were found to migrate towards sites of corneal irritation in rabbits (McCutcheon, 1946). Chemotaxis is now known to be responsible for a variety of immunological processes including the recruitment of leukocytes to sites of infection and the movement of lymphocytes around the body (reviewed in (Jin *et al.*, 2008)). Various immune cells are thought to move around the bloodstream and lymphatic system in search of pathogenic activity. Chemotactic gradients act as "pathways" leading the immune system to the source of invading pathogens (Delves and Roitt, 2000a, b). Cells can migrate along these gradients both in the bloodstream and across epithelia without damaging the intercellular tight junctions (Burns *et al.*, 2000).

One group of peptides that control chemotactic activity are the group of low molecular weight chemokines (Murdoch and Finn, 2000). These peptides have been shown to be selectively chemoattractive for leukocytes *in vitro* and elicit their effects via the binding of transmembrane G-protein-coupled receptors (Murphy, 1994). This binding elicits a signalling cascade in the target cell with transient increases in Ca^{2+}, cAMP and cGMP being observed (Parent and Devreotes, 1999). A summary of chemokine activity is shown in Figure 1.12.

Both the α- and β-defensins have been reported to have chemotactic properties at nanomolar concentrations, although the cells to which these are targeted vary. These

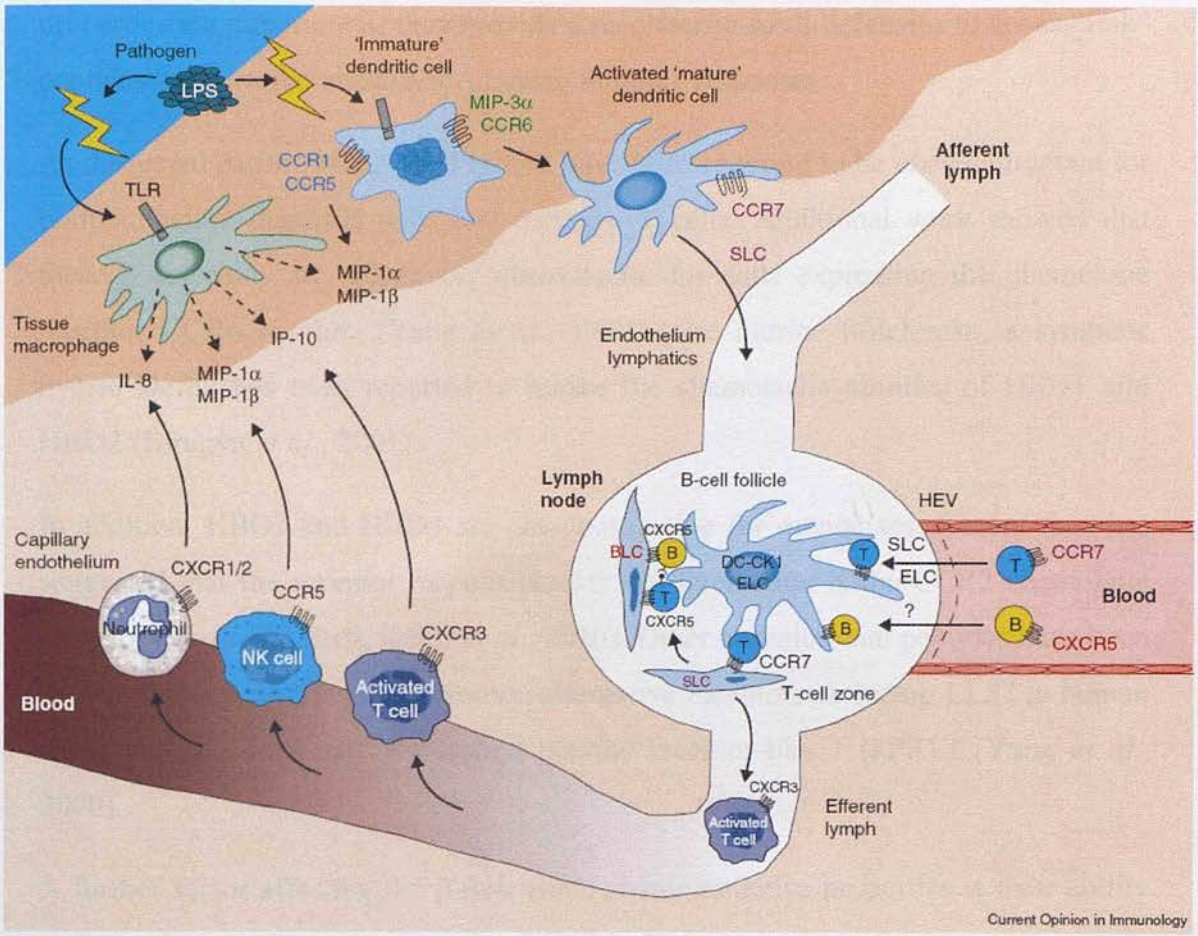


Figure 1.12 Chemokine activity (taken from Luster, 2002)

The chemokines are involved in a number of functions and coordinate the trafficking of a variety of immune cells, including dendritic cells and B and T cells. This helps mediate the immune response.

Toll-like receptors (TLRs) are activated specifically by a number of different microbial stimuli, and in turn the TLRs mediate a local release of chemokines, resulting in the chemoattraction of immature dendritic cells. This also results in the recruitment of naive T and B cells from the blood into the lymph nodes. Here, contact is made with dendritic cells and activation occurs.

properties are important as they provide a mechanism for β-defensins to act as "link" peptides between the innate and adaptive immune responses.

As discussed earlier, HBD1 and HBD2 have been reported to be chemoattractant for both immature dendritic cells and memory T cells. Additional work showed that these β-defensins are selectively chemotactic for cells expressing the chemokine receptor CCR6 *in vitro* (Yang *et al.*, 1999). The murine β-defensin, a synthetic hybrid Defb2 has been reported to mirror the chemotactic abilities of HBD1 and HBD2 (Biragyn *et al.*, 2001).

In addition, HBD3 and HBD4 are chemoattractive for monocytes. Recent data has suggested that the receptor responsible for this interaction is the CCR2 chemokine receptor (Jin *et al.*, 2010; Röhrl *et al.*, 2010). Other antimicrobial peptides have been shown to chemoattract monocytes via alternative receptors including LL37 (a human cathelicidin), which acts via formyl peptide receptor-like 1 (FPRL1)(Yang *et al.*, 2000).

A further factor affecting the β-defensin's chemoattractive properties is their ability to form dimers. A murine variant of Defb8, Defr1 was discovered to contain only five cysteine residues (Morrison *et al.*, 2002). This allowed the peptide to form dimers (Campopiano *et al.*, 2004) via the free fifth cysteine residue (which was not bound via an intramolecular disulphide bond, as in six-cysteine defensins). This peptide maintains chemoattractive properties similar to those seen with Defb8. This activity was retained even when the peptide was monomerised following DTT treatment (Taylor *et al.*, 2009). This, combined with the fact that Defb14 maintains its chemoattractive properties even when five out of its six conserved cysteines have been substituted with tyrosine (Tyrrell *et al.*, 2009) suggests that the β-defensin cysteines may only serve to stabilise the structure and perhaps perform some other function. In α-defensins, it has been suggested that the cysteines may stabilise the defensin fold in order to protect the peptides against degradation by serum proteases (Maemoto *et al.*, 2004).

1.5.5 A surprising role for β-defensins in pigment distribution

In recent years, increasingly diverse roles have been reported for the β-defensins. A paper in 2007 reported a role for canine β-defensin 3 (CBD103) in pigment type-switching in domestic dogs (Candille *et al.*, 2007).

The mechanism by which pigmentation occurs in dogs is largely conserved throughout mammals. Skin and hair colour is mainly the result of combinations of two pigments: eumelanin (brown/black) and pheomelanin (red/yellow)(reviewed in (Rees, 2003)). The ratio of synthesis of these pigments depends on a balance achieved between two principle genes: *Agouti* and *Melanocortin 1 receptor* (*Mc1r*)(Andersson, 2003; Klungland and Vage, 2003). In this system, the MC1R receptor acts like a molecular "switch" and when activated by α-Melanocyte Stimulating Hormone (α-MSH), signals via cAMP to allow for the exclusive production of eumelanin by the melanocytes. The presence of the physiological antagonist, Agouti will inhibit Mc1r activity and lead to the production of pheomelanin. This is summarised in Figure 1.13.

In 2007, it was found that dog coat colour is controlled by an additional locus (termed the "K" locus). The K locus was found to segregate with black coat colour and behaved in a dominant manner. The locus was mapped to the β-defensin cluster, where a three base pair deletion in the *CBD103* gene was detected and results in the loss of the first amino acid of the mature peptide. (Candille *et al.*, 2007). Expression of both the mutant and wild type CBD103 in mice resulted in the dominant inheritance of black coat colour, even in the presence of Agouti. It was also shown *in vitro* that CBD103, HBD1 and HBD3 bind the Mc1r receptor, leading to the proposal that the CBD103 mutant protein binds MC1R, blocking the binding of Agouti, driving eumelanin production.

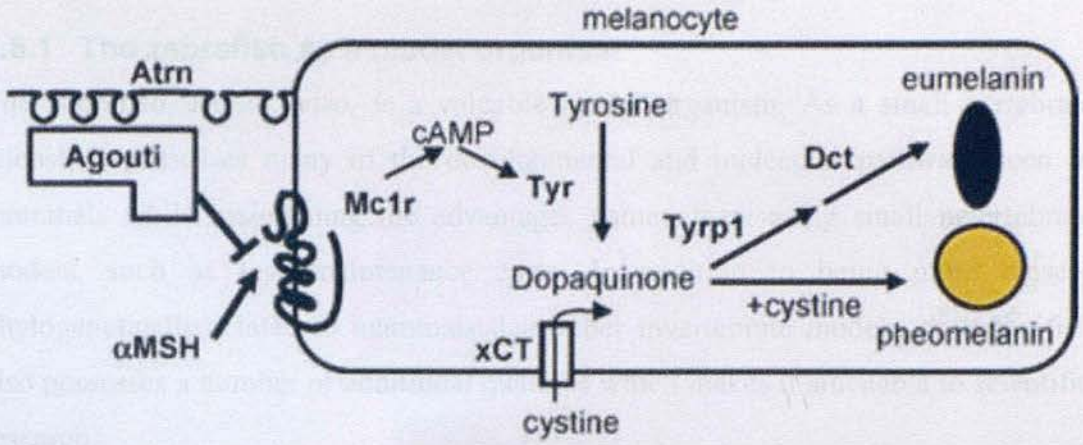


Figure 1.13 The control of pigment-type switching (taken from Candille *et al.*, 2007)

The melanocortin 1 receptor, Mc1r, is thought to maintain a basal "resting" level of activation, leading to the production of cAMP. This activates a signalling pathway, resulting in the synthesis of the black pigment, eumelanin. This pigment is also strongly induced upon the binding of Mc1r by its agonist α-MSH. However, in the presence of the Agouti protein, α-MSH is displaced and the production of pheomelanin (yellow pigment) is driven.

1.6 THE ZEBRAFISH AS A MODEL FOR β-DEFENSIN STUDIES

1.6.1 The zebrafish as a model organism

The zebrafish, *Danio rerio,* is a valuable model organism. As a small vertebrate teleost, it possesses many of the developmental and molecular pathways seen in mammals while maintaining the advantages gained from using small invertebrate models, such as low maintenance costs. In addition to being more closely phylogenetically related to mammals than other invertebrate models, the zebrafish also possesses a number of additional qualities which makes it amenable to scientific research.

Firstly, zebrafish are small in size and large numbers can be maintained in modest aquariums at a fraction of the cost of a mammalian facility. In addition, zebrafish breed very efficiently and each breeding pair can be expected to produce 200-300 embryos weekly. This provides large sample sizes for experiments, to allow greater degrees of statistical power to be achieved when analysing data.

A further benefit of employing zebrafish is their *ex utero* development (see Figure 1.14). Zebrafish embryogenesis is completed within five days of development and the permeability of early embryos means they can be treated with drugs merely by adding these to the embryonic growth medium. Early embryos can also be easily manipulated by micro-injection of DNA, RNA or morpholinos. The embryos remain transparent throughout embryogenesis, allowing structures to be examined under a light microscope, and are amenable to fluorescence imaging.

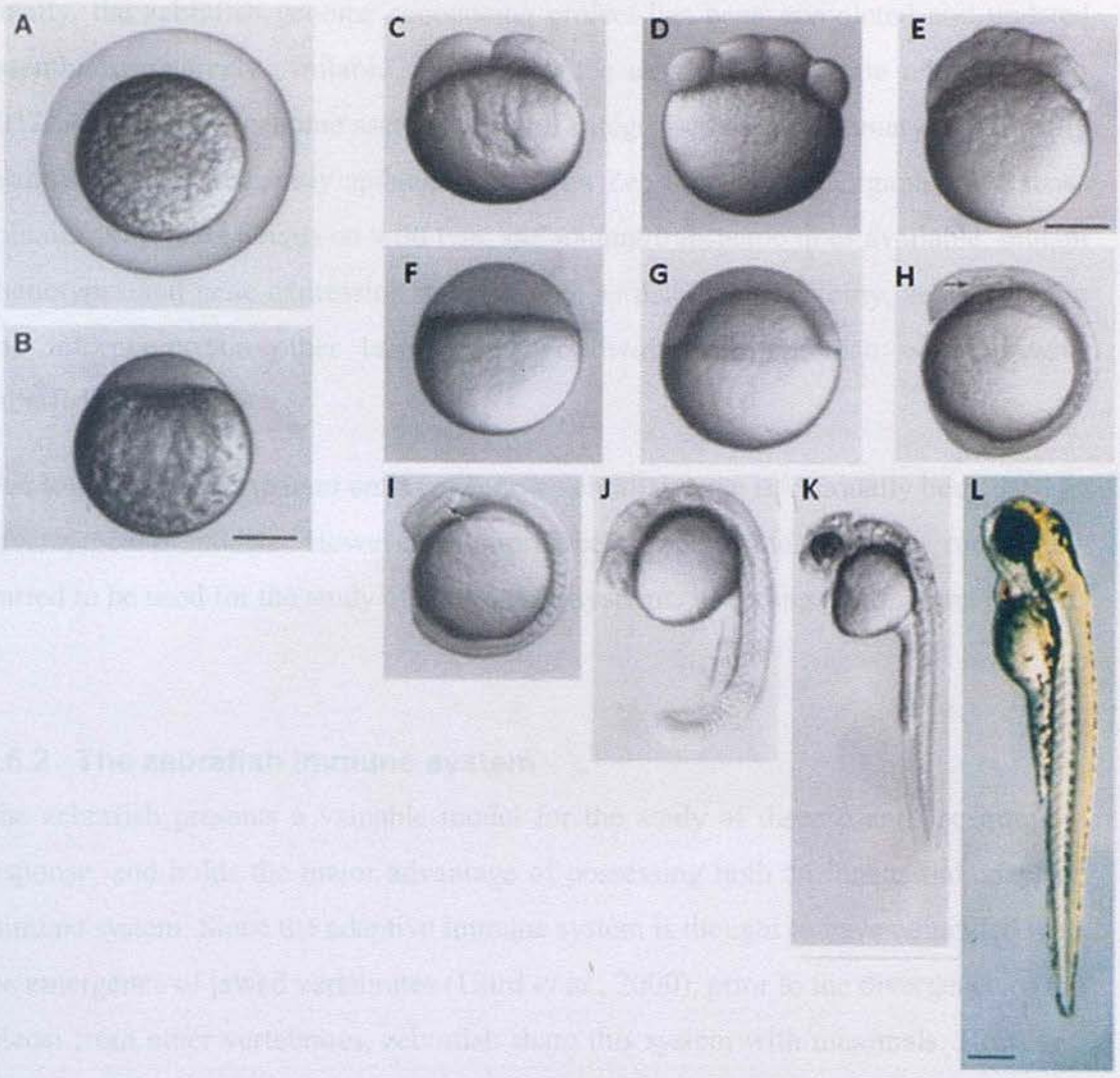


Figure 1.14 The first 24 hours of zebrafish development (adapted from Kimmel *et al.*, 1995)

The zebrafish embryo develops rapidly and *ex vivo*. Initially, the one-cell embryo (**B**) rapidly divides to form a cluster of cells on top of the yolk sac after 2.25 hours of development (**C-F**). Following this, gastrulation begins with cells migrating around the yolk, reaching the "shield" stage after approximately 6 hours (**G**). The somites then start to develop and the patterning, with a distinct eye and tail forming by 12 hours post fertilisation (hpf)(**H**). Following 24hpf (**J**), the embryo has developed sufficient nervous ability to react to external stimuli. Pigmentation occurs at approximately 28hpf (**K**) and the majority of the organs have fully developed by 72hpf (**L**).

Finally, the zebrafish genome sequencing project has been completed and updated assemblies are freely available online, with the next major genome release due in 2012. Although the genome assembly is still categorised as "preliminary", publically available data is frequently updated. The ZFIN Zebrafish Model Organism Database contains up-to-date details on wild type and mutant/transgenic lines available, mutant phenotypes and gene expression data, as well as details on anatomy, zebrafish care and information on other laboratories worldwide who are also working with zebrafish.

Due to its rapid, transparent embryogenesis, zebrafish have traditionally been used as developmental models. However in more recent years, the zebrafish model has started to be used for the study of a variety of systems, including the immune system.

1.6.2 The zebrafish immune system

The zebrafish presents a valuable model for the study of disease and the immune response, and holds the major advantage of possessing both an innate and adaptive immune system. Since the adaptive immune system is thought to have coincided with the emergence of jawed vertebrates (Laird *et al.*, 2000), prior to the divergence of the teleost from other vertebrates, zebrafish share this system with mammals. However, the zebrafish has the additional advantage of functioning only with basic innate immunity up until at least 4dpf (days post fertilisation) when the first adaptive immunity markers become detectable (Allen and Neely, 2010). This presents the opportunity to study the innate immune system independently of the adaptive within the embryonic and larval zebrafish.

In order to comprehensively display the variety and complexity of the zebrafish immune system, a comparison of the zebrafish and human systems is depicted in Figure 1.15. A number of studies of zebrafish haematopoiesis have revealed that the majority of human immunity cells have corresponding zebrafish cells, although the functionality of all these cells has not yet been established.

As shown in Figure 1.15, the zebrafish immune system possesses a well-developed complement system with conserved inflammatory proteins including TNFα, various

interleukins and other chemokines. In addition, the circulating cells include neutrophils and macrophages, both of which have been genetically engineered as GFP fusion proteins, which can be easily visualised in early embryos (Lawson and Weinstein, 2002; Mathias *et al.*, 2006).

The zebrafish has been used as a model to study a number of diseases, however the modelling of *Mycobacterium* infections (first described in Prouty *et al.*, 2003) is particularly relevant. Utilising *M. marinum* species, it was shown that zebrafish form caseating granulomas resembling those seen in humans, following infection. Strikingly, the pathology of this disease in zebrafish has been claimed to be a better model of human tuberculosis than mouse (Phelps and Neely, 2005). In more recent work, a comparison between *M. marinum* and *M. tuberculinum* (human pathogen) revealed significant conservation of host-pathogen interactions (Tobin and Ramakrishnan, 2008), highlighting the relevance of the model to tuberculosis studies. A recent study also marked the importance of the zebrafish model to tuberculosis studies when the study of granulomas caused by *M .tuberculinum* revealed a novel mechanism by which mycobacteria can induce the formation of granulomas to facilitate growth (Volkman *et al.*, 2010).

Zebrafish also possess several additional immune components that make them viable for the study of β-defensin function: they have been reported to express homologues of both CCR6 (Liu *et al.*, 2009) and its ligand CCL20, as well as an array of Toll-like receptors. This provides the potential for the study of defensin-induced chemotaxis, as well as the induction of the immune system via the Toll-like receptors. Interestingly, the existence of three β-defensin-like genes showing expression in the adult zebrafish have also been reported (Zou *et al.*, 2007).

1.6.3 The zebrafish defensins

Zou *et al.*, 2007 reported the expression of three zebrafish β-defensin-like genes, *defbl1*, *defbl2* and *defbl3* in various adult tissues, along with a number of additional teleost species. The zebrafish defensins were found to contain the six cysteine

	Human	Zebrafish
General		
Lymphatic system	ZF has no lymph nodes but does have putative lymphatic vessels including a thoracic duct	
Complement system	Well developed in ZF with shared human elements	
Inflammatory proteins	Well conserved between human and ZF, i.e., TNFα, NF-κB, COX2, IL-1, IL-8 and other C-C and C-X-C chemokines	
MHC	Class I, II, III all present in ZF	
Myeloid		
Neutrophils		
Nuclear lobes	4–5	2–3
Cytoplasm	Heterophilic with azurophilic and non-azurophilic granules similar to human	
Motility	+	+
Phagocytic activity	+	+
Eosinophils		
Morphology	Morphologically distinct from human counterpart	
Monocytes/macrophages		
Motility	+	+
Phagocytic activity	+	+
Respiratory burst	+	+
Ability to activate T/B cells	+	+
Mast cells and basophils	Currently not characterized in ZF	
Lymphoid		
T cells		
Site of initial development	Bone marrow	Kidney marrow
Educated in thymus	+	+
TCR	ZF TCRα, β, δ, γ chains identified [http://www.ncbi.nlm.nih.gov/Genbank/index.html]	
Rag-dependent V(D)J rearrangement	+	+
Gene expression	Similarities include *ikaros*, *lck*, *GATA-3*, *rag*	
B cells		
Embryonic development	Initially develop in ZF pancreas	
Ig subtypes	A,D,G,E, M	D, M, Z
Rag-dependent V(D)J	+	+

Figure 1.15 A comparison of the human and zebrafish immune systems (taken from Meeker and Trede, 2008)

This comparative table illustrates the vast similarities between the human and zebrafish immune systems. Almost every area of the human system has a zebrafish counterpart, with the fish system displaying a well-developed innate and immune response.

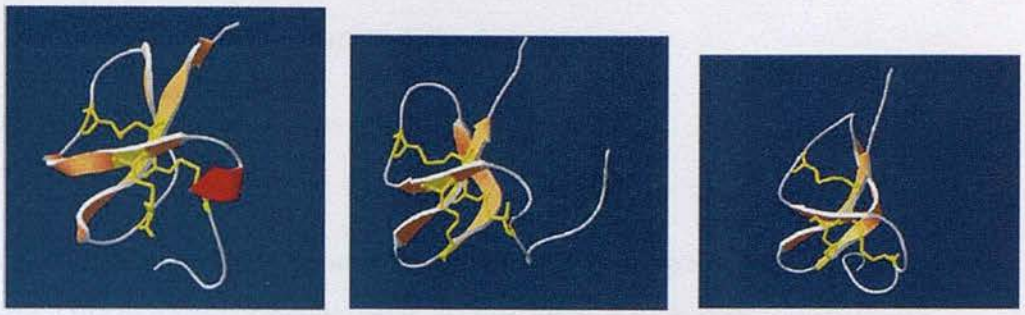
residues typical of the defensin family and following computational analysis, were predicted to fold to form three β-sheets, adopting a folding pattern similar to that of the β-defensins in plants and mammals (Zou *et al.*, 2007)(Figure 1.16a).

In addition, the zebrafish defensins were shown to share a similar synteny to the human β-defensin locus at 8p23.1; *defbl1* and *defbl2* are both located at the same locus on chromosome 21, between SPAG11 and the olfactory pseudogene OR7E157P, as is seen in the human genome (Figure 1.16b).

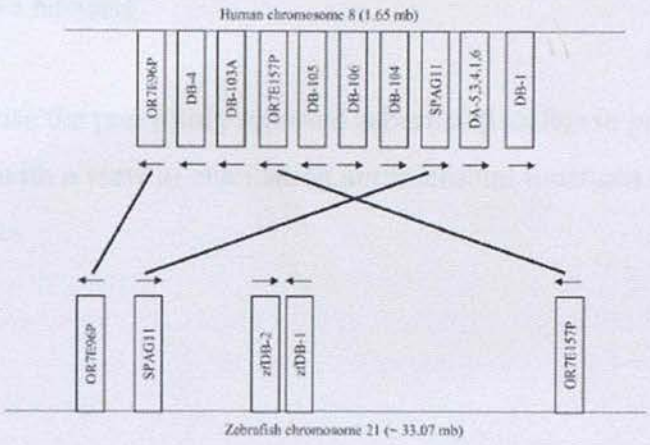
The expression of the zebrafish defensins was originally shown to be varied, with *defbl1* and *defbl3* being detected in an number of adult tissues including the gut, skin and gills, and *defbl2* only in the gut (Zou *et al.*, 2007, Figure 1.16c). Subsequent work has reported an increasing anterior-posterior gradient of zebrafish defensin expression along the adult gut, along with more specific expression of *defbl1* in the larval skin and swim bladder (Oehlers *et al.*, 2011).

Beyond these initial studies, however, no functional characterisation of the zebrafish defensins has been carried out.

(a)



(b)



(c)

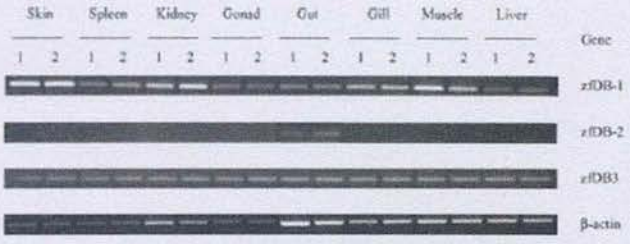


Figure 1.16 The zebrafish β-defensin-like genes (taken from Zou *et al.*, 2007)

(a) Computational analysis predicted that *defbl1*, *defbl2* and *defbl3* fold similarly to the mammalian β-defensins, with apparent conservation of the triple-stranded antiparallel β-sheet observed.

(b) The synteny surrounding *defbl1* and *defbl2* on zebrafish chromosome 21 shows some conservation with the human 8p23.1 defensin cluster.

(c) Expression analysis using RT-PCR revealed the differential expression of the zebrafish defensins. *defbl1* and *defbl3* were detected in all tested adult tissues, whereas *defbl2* was detected only in the gut.

1.7 THESIS AIMS

In this thesis, I have investigated the function of vertebrate defensins, by looking in detail at specific zebrafish and murine defensins.

The broad aims of the thesis are as follows:

1. To observe how both the primary and tertiary structure of murine Defb14 affect the peptide's antimicrobial activity against both gram positive and gram negative bacteria.

2. To characterise the previously reported zebrafish β-defensin peptides, particularly with a view to elucidating antimicrobial functions and any other potential roles

Chapter 2: The Structure-Activity Relationship of Murine Defb14

"Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life itself"

Francis Crick

2.1 PREFACE

The emergence of antibiotic-resistant pathogens is a growing concern due to our ever-increasing dependence on antibiotics in medicine, veterinary medicine and agriculture. A number of classes of antibiotic were discovered in a "golden era" of antibacterial drug discovery beginning in the 1940s, and pathogens were unable to build up a robust resistance to the vast array of drugs being produced. However a combination of more widespread use of antibiotics and the difficulties associated with bringing novel drugs to market and their subsequent financial viability has led to a decrease in the number of novel antibiotics being developed. In a 2004 report, the World Health Organisation (WHO) identified antibiotic resistant bacteria as the largest worldwide pharmaceutical gap (Kaplan and Laing, 2004).

The 1950s and 1960s saw the development of a huge number of novel antibiotics, belonging to a number of different chemical categories (Coates *et al*, 2002; Wenzel 2004). Different categories of antibiotic target different bacterial processes, for example the β-lactams inhibit bacterial cell wall synthesis (Izaki *et al*, 1968), whereas the aminoglycosides and tetracyclines target protein synthesis (Brock, 1963). The possession of antibiotics which target different processes is an important strategy in combating bacteria which have mutated to develop resistance. However, since the 1960s, only two new categories of antibiotic have been developed; the oxazolidinones (Linezoid, first approved in 2001)(Moellering ,2003) and a cyclic lipopeptide Daptomycin (first approved in 2003)(Woodworth *et al*, 1992). Instead, novel antibiotics since the 1960s have largely consisted of derivatives of the existing classes of antibiotics.

The use of peptides derived from the innate immune system, such as the β-defensins, represents a potential source from which to develop novel antibiotics. Not only are the peptides small (which translates to lower production costs and faster synthesis), but their mechanism of action is general, targeting the entire bacterial cell membrane of both gram positive and negative species. This presents an inviting economic model, as the non-specific nature lowers the risk of bacterial resistance emerging quickly, since this would require major changes to the entire cell membrane structure (as opposed to the mutation of a single protein for more specific drugs). In addition,

the β-defensins have the further advantage of being largely bactericidal, as opposed to many other conventional antibiotics which are bacteriostatic. There is some data that suggests that bactericidal drugs are more effective *in vivo* and correlate to higher survival rates for endocarditis than bacteriostatic (Finberg, 2004). On an economic level the β-defensins are inviting, as bactericidal activity presents less of an opportunity for bacteria to evolve resistance. This effectively extends the "shelf-life" of the drug, making it a more attractive option for development.

The defensins are such a great area of interest that two new defensin-inspired drugs have been produced and have progressed through to the clinical trials stage of development. Encouragingly, in September 2010, PolyMedix Inc began Phase 2 clinical trials of their new compound PMX-30063 (Scott *et al.*, 2008), a synthetic defensin biomimetic which targets both gram positive and negative bacteria. This drug has the financial advantage of being non-protein (the molecule is based on a carbon backbone), as well as crucially being tolerated systemically.

A second defensin-like antibiotic in clinical trials is Novozymes and Sanofi Aventis's Plectasin NZ2114 (Ostergaard *et al.*, 2009). This peptide is derived from the fungus *P.nigrella* and has been selected as a high performing variant against vancomycin-resistant *S.aureus* (*VRSA*). This peptide has already had promising results in a Phase I clinical trial, where it has been shown to be tolerated at high doses.

In order to investigate defensin-derived peptides which might be of further clinical relevance, research emphasis has been placed on the analysis of the origin of defensin antimicrobial activity. Although the underlying basis of defensin function is still not yet known, investigations have been carried out to elucidate the effects of altering both the structure and charge of antimicrobial peptides.

A number of studies focussed on the role of the characteristic cysteine:cysteine disulphide bonding in antimicrobial activity. Wu *et al.*, 2003, used synthetic analogues of human β-defensin 3 (HBD3) to change the intramolecular disulphide bonding pattern and showed that it was not necessary for antimicrobial activity. Moreover, an analogue containing no cysteines also remained antimicrobially active.

Hoover *et al.*, 2003 reported similar findings and also showed that a fully linear HBD3 peptide retained its antimicrobial activity.

Since full cysteine connectivity does not appear to be vital for antimicrobial activity, studies have also concentrated on the role of charge and structure in activity. Hoover *et al.*, 2003 showed that fragments of the N- and C- termini of HBD3 were bactericidal to gram positive and negative bacteria, respectively. The tested fragments were all positively charged and those with a higher charge were shown to have higher antimicrobial activity. However, there was no correlation between changes in charge and the observed differential activity against bacteria of different gram stains, leading to the conclusion that although charge is important for activity, structure must play a role too (Hoover *et al.*, 2003). Krishnakumari *et al.*, 2006 also demonstrated the importance of structure when they showed that three synthetic C-terminal fragments of HBD1, HBD2 and HBD3 retained antimicrobial activity against both gram positive and negative bacteria when in their dimeric forms. However, when the peptides were reduced, they only retained activity against gram negative *E.coli*. Circular dichroism spectra showed that the dimeric peptides retained structures similar to the native peptide, whereas the reduced monomeric spectra indicated a loss of structure, which was suggested to be responsible for the reduced antimicrobial potential (Krishnakumari *et al.*, 2006).

While an abundance of studies have focussed on the human β-defensins, their murine orthologues hold the added potential for *in vivo* studies. Although they can provide insight into the molecular function of antimicrobial peptides, *in vitro* antimicrobial assays are taken entirely out of biological context. The *in vitro* study of murine defensins, however, could lead to the discovery of highly active peptides or peptide fragments whose findings could then be applied directly to relevant *in vivo* studies.

Previous work in this group investigated the antimicrobial activity of murine Defb8, finding it to be poor against all tested species. However, Defr1, an allele of Defb8 in C57B1/6 mice was shown to be strongly antimicrobial (Morrison *et al.*, 2002). The sequences of the two alleles are shown in Table 2.1.

Peptide	Sequence	Antimicrobial?
Defr1	DPVTYIRNGGICQYRCIGLRHKIGTCGSPFKCCK	+++
Defb8	EPVSCIRNGGICQYRCIGLRHKIGTCGSPFKCCK	-

Table 2.1 Sequences of Defr1 and Defb8 peptides (data from Morrison *et al.*, 2002)

Although the sequences of these alleles only differ by three amino acids, the antimicrobial activity of Defr1 has potent antimicrobial activity, whereas Defb8 does not.

It was shown that due to its odd number of cysteine residues, the native state of Defr1 was as a covalent dimer, while Defb8 is monomeric. The restoration of six cysteines to Defr1 (resulting in monomerisation) leads to the abrogation of antimicrobial activity (Taylor *et al.*, 2007), indicating that dimerisation was responsible for the increased antimicrobial activity in this peptide. This finding, along with the similar findings of Krishnakumari *et al.*, 2006 in HBD1-3 led to the decision to investigate the role of dimerism in antimicrobial activity in murine Defb14.

Defb14 is the murine orthologue of HBD3 (Semple *et al.*, 2006) and is a highly effective, salt-insensitive bactericidal agent against an array of both gram positive and negative bacteria. Defb14 is monomeric, so a synthetic analogue (Defb14-1C^{V}) containing just one cysteine residue at canonical position V was produced, to determine whether a dimeric peptide would show even better antimicrobial activity (Figure 2.1a, Taylor *et al.*, 2008). Dimerisation did not improve the activity of full length Defb14-1C^{V}, however an investigation into the antimicrobial activity of Defb14-1C^{V}-inspired peptide fragments indicated that the amino acids responsible for antimicrobial activity in this molecule reside in the N-terminal (Figure 2.1b). In further work, an additional two active fragments of interest, Defb14-1C^{V}(1-10) and Defb14-1C^{V}(6-17) (Figure 2.2) were identified (Reynolds *et al.*, 2010), following screening of a number of N-terminal fragments for antimicrobial activity against a panel of gram negative and positive bacteria.

a

Peptide	Sequence	charge
HBD3-1c^{V}	GIINTLQKYY**A**RVRGGR**A**AVLS**A**LPKEEQIGK**A**STRGRK**CA**RRKK	+11 (+22)
HBD3	GIINTLQKYY**C**RVRGGR**C**AVLS**C**LPKEEQIGK**C**STRGRK**CC**RRKK	+11
	** * ** ******** ** ****** ** ****** **	
Defb14	FLPKTLRKFF**C**RIRGGR**C**AVLN**C**LGKEEQIGR**C**SNSGRK**CC**RKKK	+12
Defb14-1c^{V}	FLPKTLRKFF**A**RIRGGR**A**AVLN**A**LGKEEQIGR**A**SNSGRK**CA**RKKK	+12 (+24)
Defb14-1c^{I}	FLPKTLRKFF**C**RIRGGR**A**AVLN**A**LGKEEQIGR**A**SNSGRK**AA**RKKK	+12
Defb14-0c	FLPKTLRKFF**A**RIRGGR**A**AVLN**A**LGKEEQIGR**A**SNSGRK**AA**RKKK	+12
D14ip1	FLPKTLRKFF**A**RIRGGR**A**AVLN**A**	+6
D14ip2	LGKEEQIGR**A**SNSGRK**AA**RKKK	+6
D14ip3	LGKEEQIGR**A**SNSGRK**CA**RKKK	+6 (+12)
D14ip7	IRGGR**A**AVLN**A**LGKEEQIGR**A**S	+2

b

Organism	Minimum bactriocidal concentration								
	HBD3	HBD3-1c	Defb14	Defb14-1c^{V}	Defb14-0c	D14ip1	D14ip2	D14ip3	D14ip7
					μg/ml				
P. aeruginosa PAO1	1.5	1.5	1.5 (1.5)	1.5 (1.5)	1.5	1.5	>50	>50	>50
S.aureus ATCC 25923	3.13	3.13	3.13	3.13	3.13	3.13	>50	>50	>50

Figure 2.1 The antimicrobial activity of Defb14 inspired peptides (adapted from Taylor *et al.*, 2008)

Defb14 inspired peptides were tested for antimicrobial activity against gram negative *P.aeruginosa* and gram positive *S.aureus*. **a** depicts the sequences of the tested peptides and shows the homology between HBD3 and Defb14. The six conserved cysteine residues and their substituted alanine residues are shown in bold.
b shows the minimum bactericidal concentrations (MBC) (the minimum concentration required to kill 99.99% initial inoculums) for the synthetic peptide panel. A ">" value indicates no antimicrobial activity was detected. Defb14-1C^{V} shows no increased antimicrobial activity over Defb14 for either *P.aeruginosa* or *S.aureus*. The activity of the N-terminal peptide D14ip1

Organism and strain	MBC (μM) for peptide[a]:							
	Defb14	Defb14-1C^{V}	Defb14-1C^{V} (1-23)	Defb14-1C^{V}Δ (1-23)	Defb14-1C^{V} (1-10)	Defb14-1C^{V} (6-17)	Defb14-1C^{V} (14-23)	Defb14-1C^{V} (13-34)
Gram negative								
P. aeruginosa PAO1	0.3	0.3	0.6	>20.5	1.2	1	>48	>22
A. baumannii ATCC 19606	0.6	0.6	1.2	>20.5	19	1	>48	>22
Burkholderia cenocepacia J2315	>20	>20	>20	>20.5	>40	>33	>48	>22
E. coli ATCC 25922	0.6	0.6	1.2	>20.5	9.6	2	>48	>22
Gram positive								
E. faecalis ATCC 700802	1.2	1.2	2.4	>20.5	19	16.5	>48	>22
S. aureus ATCC 25923	0.6	0.6	1.2	>20.5	38.6	9.6	>48	>22
MRSA J2918	1.2	1.2	2.4	>20.5	38.6	4.8	>48	>22

Figure 2.2 The antimicrobial activity of Defb14 inspired peptide fragments against a panel of gram positive and negative bacteria (taken from Reynolds *et al.*, 2010)

Antimicrobial activity of a variety of N-terminal Defb14 fragments was tested. The shaded bar beneath the peptide name indicates its position within the parent Defb14-1C^{V} molecule. Antimicrobial activity is denoted as an MBC value, given in μM. A value of ">" indicates no antimicrobial activity was detected.
The N-terminal half of the peptide Defb14-1C^{V}(1-23) showed potent antimicrobial activity, comparable with that of the parent peptide, however the C-terminal half and peptides Defb14-1C^{V}(14-23) and Defb14-1C^{V}(13-34) showed no detectable activity. Defb14-1C^{V}(1-10) and Defb14-1C^{V}(6-17) were identified as active fragments.

In this chapter I continue the analysis of the bactericidal activity of Defb14-1C^{V} and investigate further the origin of its activity using a library of progressive truncated peptides. The use of a deletion series allows for the continuation of investigation into the activity of the peptide fragments, but within the context of a full length molecule. I look at the effects of dimerisation, charge and primary sequence on the bactericidal activity against a panel of gram positive and negative bacteria. In order to introduce higher clinical relevance to the study, the panel of bacteria tested included strains relevant to the current crisis of antibiotic resistance, including *MRSA*, *A.baumannii* and *E.coli*.

Sections 2.3.1 – 2.3.5 of this chapter were published in Reynolds et al., 2010 and Tyrrell et al., 2009. Appropriate permissions were obtained prior to including the work in this thesis and the manuscripts for these publications can be found in Appendix 2.

2.2 AIMS

The aims of this work were to:

1. Assess the bactericidal properties of a panel of Defb14-inspired synthetic peptide fragments in order to further dissect the residues important for activity
2. Observe and identify the regions of Defb14 critical for bactericidal activity
3. Further investigate the effect of dimerisation on bactericidal activity
4. Investigate the bactericidal activity of the peptide panel under more physiological conditions containing serum and salt
5. Utilise the data from these analyses to draw conclusions about the role of charge in antimicrobial activity

2.3 ANTIMICROBIAL ACTIVITY OF A DEFB14-INSPIRED PEPTIDE DELETION SERIES

Synthetic Defb14-inspired peptide fragments containing sequential peptide deletions of three amino acids were obtained (Derek Macmillan, UCL), as shown in Table 2.2. These fragments were based on the Defb14-1C^{V} peptide which has all its cysteines (apart from cysteine V) mutated to alanine residues. This was done to enable the formation of dimeric peptides (via disulphide bridges between the remaining cysteine residues) and has previously been shown to have no effect on the parent peptide's antimicrobial activity (Taylor *et al.*, 2008).

The activity of the peptide deletion series was determined by recording the minimum bactericidal concentration (MBC) for each of the peptides against a panel of gram positive and negative bacteria at 37°C. This peptide concentration is defined as the minimum concentration required to kill 99.99% bacteria incubated with the peptide and is therefore of high clinical relevance. An alternative test which could have been employed is the minimum inhibitory concentration (MIC). The MIC determines the minimum concentration of peptide at which bacterial growth is inhibited and thus bacteriostatic compounds would yield a positive result. In a clinical setting, bacteriostatic antibiotics will prevent bacterial growth until the infection can be managed by the host immune system. However, such compounds are not effective in treating immunodeficient patients or for the treatment of infections affecting immune-incompetent regions (for example, meningitis)(Booth, 2001), in which case a bactericidal agent is necessary. For this reason, MBC values were determined for all tested compounds.

2.3.1 The bactericidal efficiency of the monomeric Defb14 deletion series varies according to bacterial gram status

During synthesis, the deletion peptides spontaneously form dimers due to their single free cysteine residue. The deletion series was reduced with dithiothreitol (DTT) to produce monomeric peptides which maintained this state throughout the assay (verified by mass spectrometry, Martin De Cecco, data not shown). As the amino acid deletions progress along the N-terminal of Defb14-1C^{V}, the peptide MBCs

Peptide	Sequence	Charge (monomer)	Charge (dimer)	ΔG
Defb14	FLPKTLRKFFCRIRGGRCAVLNCLGKEEQIGRCSNSGRKCCRKKK	+12	N/A	-11.51
Defb14-1C^{V}	FLPKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+12	+24	-13.56
Def14-1C^{V}Δ(1)	LPKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+12	+24	-14.21
Def14-1C^{V}Δ(1-2)	PKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+11	+22	-15.25
Def14-1C^{V}Δ(1-5)	LRKFFARIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+10	+20	-13.67
Def14-1C^{V}Δ(1-8)	FFARIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+8	+16	-12.43
Def14-1C^{V}Δ(1-11)	RIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+8	+16	-14.52
Def14-1C^{V}Δ(1-14)	GGRAAVLNALGKEEQIGRASNSGRKCARKKK	+6	+12	-13.21
Def14-1C^{V}Δ(1-17)	AAVLNALGKEEQIGRASNSGRKCARKKK	+5	+10	-12.38
Def14-1C^{V}Δ(1-23)	LGKEEQIGRASNSGRKCARKKK	+5	+10	-11.94
Def14-1C^{V}(1-23)	FLPKTLRKFFARIRGGRAAVLNA	+7	N/A	-1.62
Def14-1C^{V}(1-10)	FLPKTLRKFF	+4	N/A	1.13
Def14-1C^{V}(6-17)	LRKFFARIRGGR	+5	N/A	-2.06
Def14-1C^{V}(18-23)	AAVLNA	0	N/A	-0.44
Def14-1C^{V}(14-23)	RGGRAAVLNA	+2	N/A	-2.08
Def14-1C^{V}(13-34)	IRGGRAAVLNALGKEEQIGRAS	+2	N/A	-7.64

Table 2.2 The Defb14-1C^{V}-inspired peptide deletion series (amended from Reynolds *et al.*, 2010) The above table displays the sequence and charge of the Defb14-1C^{V} deletion series and a number of fragments of Defb14-1C^{V}. The six conserved cysteine residues of Defb14 are coloured in red, as the alanine substitutions at the cysteine positions 1,2,3,4 and 6 in the deletion series peptides. Hydrophobicity (ΔG) was calculated for monomers using the Wimley and White scale. Greater hydrophobicity is indicated by a larger value. Peptides were synthesised by Derek Macmillan, UCL.

increase, indicating a reduction in bactericidal activity (higher concentrations of peptide are required to maintain the same level of killing).

The data indicates that the effect of the deletions varies dependent on the gram status of the strains being tested, with the peptides' activity against gram negative bacteria being largely more robust than that against gram positive bacteria. This effect can be observed by noting how much of the parent peptide can be deleted before antimicrobial activity is lost.

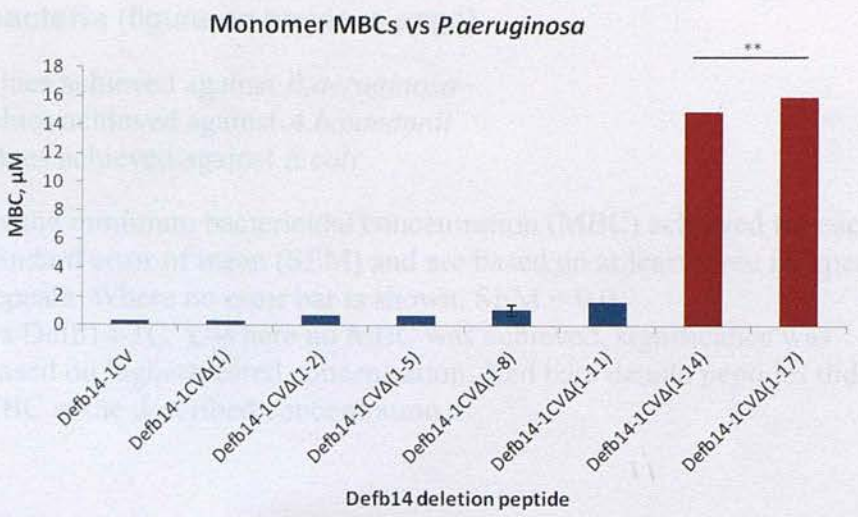
In the gram negative species, *P.aeruginosa* and *A.baumannii*, no MBC could be determined at any tested peptide concentration with the fragment Defb14-1C^{V}Δ(1-14), indicating that significant bactericidal activity is lost (Figure 2.3a,b). This is a striking contrast to the Defb14-1C^{V}Δ(1-11) peptide, which achieves an MBC 1.7 (±0.0) μM and 2.5 (±0.0) μM against *P.aeruginosa* and *A.baumannii* respectively. In contrast, a broader effect is seen in the tested gram positive species; notably an MBC was not achieved at any tested concentration of Defb14-1C^{V}Δ(1-11) in *MRSA*, and the MBC achieved for the same peptide against *S.aureus* was significantly higher than the parent peptide (Figure 2.4a,b). In addition, a more pronounced effect is seen in the analysis of *E.faecalis*, which is not killed by deletion peptides beginning with Defb14-1C^{V}Δ(1-8) (Figure 2.4c).

However, the trend of increased robustness of peptides against gram negative bacteria is not clear cut, as illustrated by *E.coli*, which is unaffected following the deletion of just the first eight amino acids (Defb14-1C^{V}Δ(1-8), Figure 2.3c).

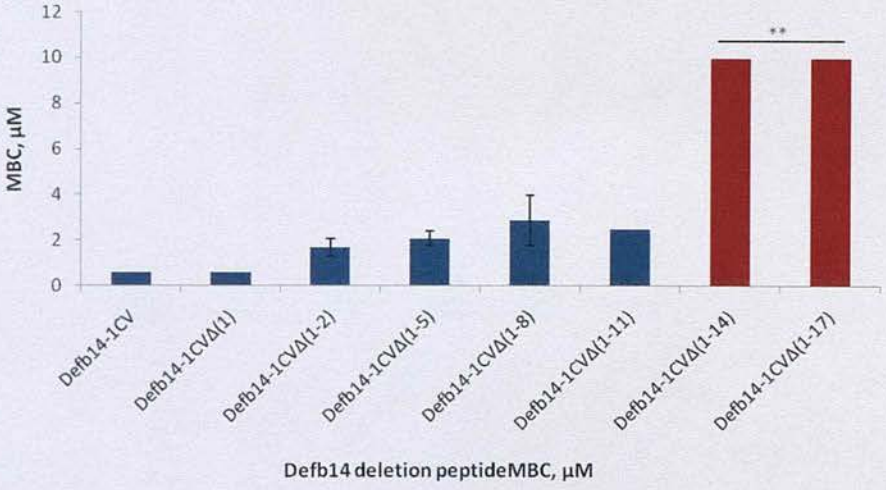
2.3.2 Dimerisation does not improve bactericidal activity in full length Defb14-1C^{V}, but preserves the activity of the deletion series

Although previous work showed no improvement in bactericidal activity of Defb14-1C^{V} in comparison to Defb14 (Taylor *et al.*, 2008), allowing the Defb14-1C^{V} peptide deletion series to dimerise has a dramatic effect on their bactericidal activity against both gram positive and negative strains. Strikingly, bactericidal activity is fully recovered for both dimeric Defb14-1C^{V}Δ(1-14) and Defb14-1C^{V}Δ(1-17) against *P.aeruginosa* and *A.baumannii*. As shown in Figure 2.5, the Defb14-1C^{V}Δ(1-14)

a


Monomer MBCs vs *P.aeruginosa*
MBC, µM
18
16
14
12
10
8
6
4
2
0
**
Defb14-1CV
Defb14-1CVΔ(1)
Defb14-1CVΔ(1-2)
Defb14-1CVΔ(1-5)
Defb14-1CVΔ(1-8)
Defb14-1CVΔ(1-11)
Defb14-1CVΔ(1-14)
Defb14-1CVΔ(1-17)
Defb14 deletion peptide

b


Monomer MBCs vs *A.baumannii*
MBC, µM
12
10
8
6
4
2
0
**
Defb14-1CV
Defb14-1CVΔ(1)
Defb14-1CVΔ(1-2)
Defb14-1CVΔ(1-5)
Defb14-1CVΔ(1-8)
Defb14-1CVΔ(1-11)
Defb14-1CVΔ(1-14)
Defb14-1CVΔ(1-17)
Defb14 deletion peptideMBC, µM

c

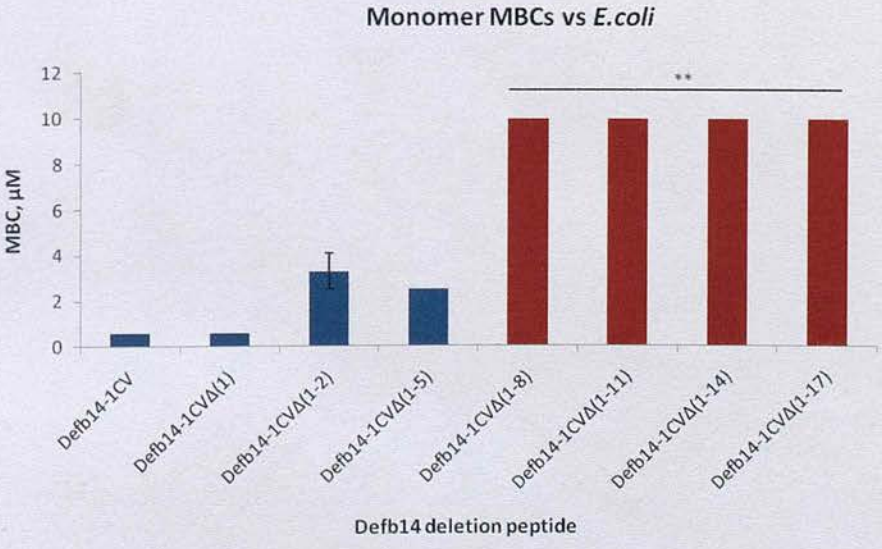
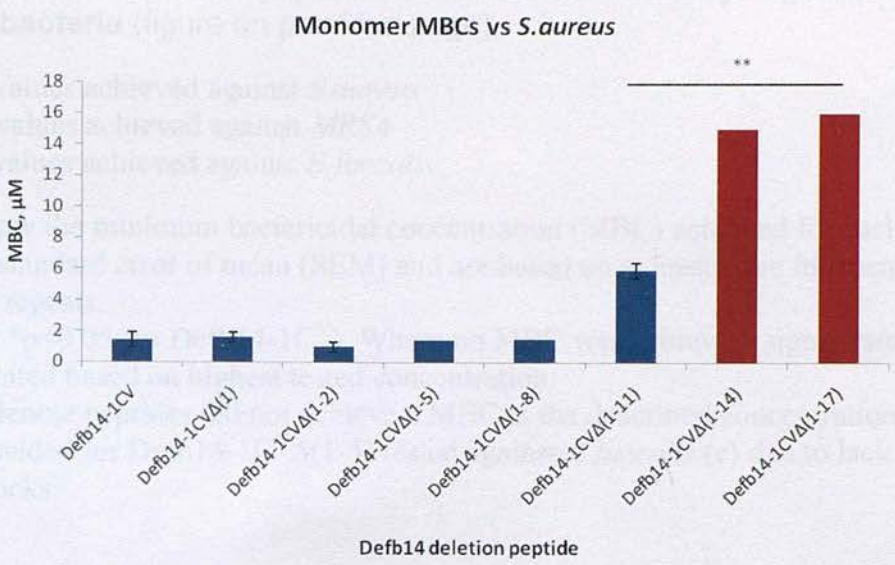

Monomer MBCs vs *E.coli*
MBC, µM
12
10
8
6
4
2
0
**
Defb14-1CV
Defb14-1CVΔ(1)
Defb14-1CVΔ(1-2)
Defb14-1CVΔ(1-5)
Defb14-1CVΔ(1-8)
Defb14-1CVΔ(1-11)
Defb14-1CVΔ(1-14)
Defb14-1CVΔ(1-17)
Defb14 deletion peptide

Figure 2.3 Monomeric peptide antimicrobial activity against gram negative bacteria (figure on previous page)

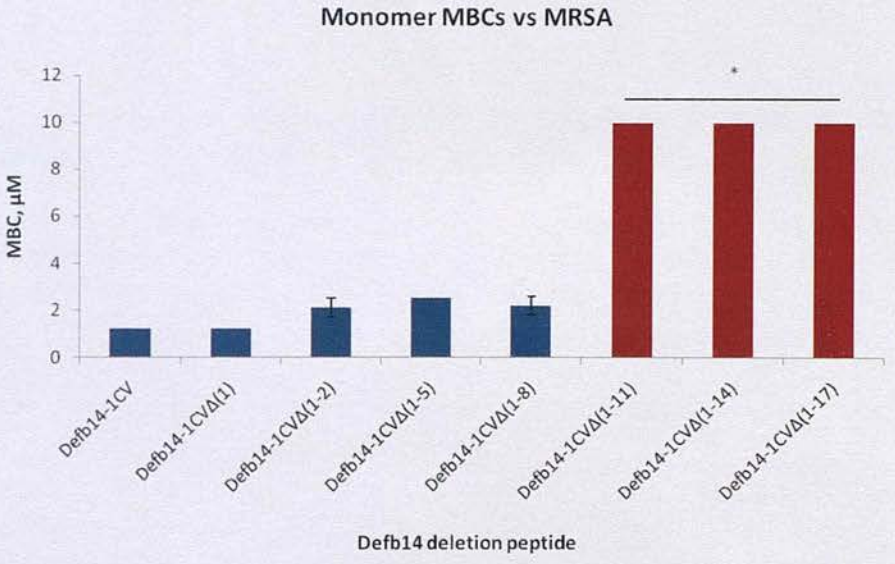
(a) MBC values achieved against *P.aeruginosa*
(b) MBC values achieved against *A.baumannii*
(c) MBC values achieved against *E.coli*

Graphs show the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM) and are based on at least three independent biological repeats. Where no error bar is shown, SEM = 0.0.
**$p<0.01$ (vs Defb14-1C^{V}). Where no MBC was achieved, significance was calculated based on highest tested concentration. Red bars denote peptides did not achieve a MBC at the described concentration

a


Monomer MBCs vs S.aureus
MBC, µM
18
16
14
12
10
8
6
4
2
0
**
Defb14-1CV
Defb14-1CVΔ(1)
Defb14-1CVΔ(1-2)
Defb14-1CVΔ(1-5)
Defb14-1CVΔ(1-8)
Defb14-1CVΔ(1-11)
Defb14-1CVΔ(1-14)
Defb14-1CVΔ(1-17)
Defb14 deletion peptide

b


Monomer MBCs vs MRSA
MBC, µM
12
10
8
6
4
2
0
*
Defb14-1CV
Defb14-1CVΔ(1)
Defb14-1CVΔ(1-2)
Defb14-1CVΔ(1-5)
Defb14-1CVΔ(1-8)
Defb14-1CVΔ(1-11)
Defb14-1CVΔ(1-14)
Defb14-1CVΔ(1-17)
Defb14 deletion peptide

c

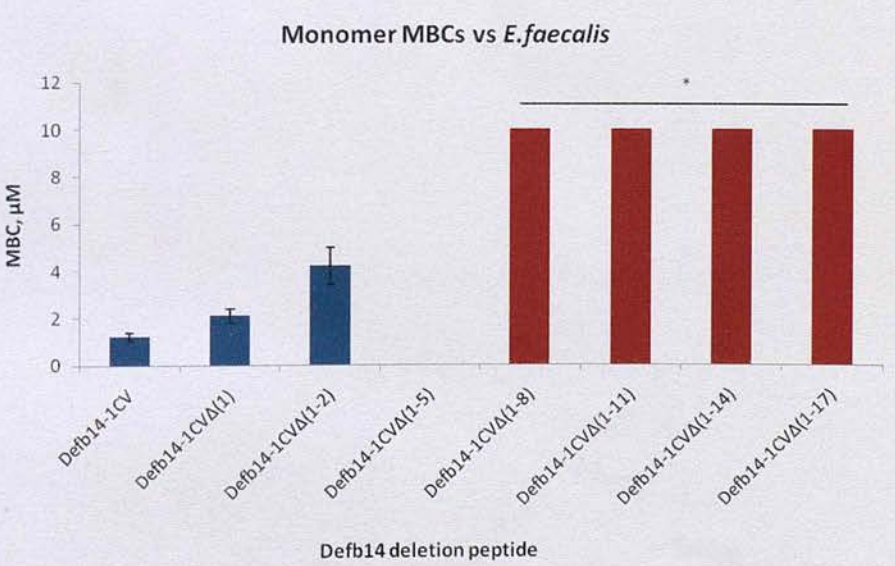

Monomer MBCs vs E.faecalis
MBC, µM
12
10
8
6
4
2
0
*
Defb14-1CV
Defb14-1CVΔ(1)
Defb14-1CVΔ(1-2)
Defb14-1CVΔ(1-5)
Defb14-1CVΔ(1-8)
Defb14-1CVΔ(1-11)
Defb14-1CVΔ(1-14)
Defb14-1CVΔ(1-17)
Defb14 deletion peptide

Figure 2.4 Monomeric peptide antimicrobial activity against gram positive bacteria (figure on previous page)

(a) MBC values achieved against *S.aureus*
(b) MBC values achieved against *MRSA*
(c) MBC values achieved against *E.faecalis*

Graphs show the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM) and are based on at least three independent biological repeats.
**p<0.01, *p<0.05 (vs Defb14-1C^{V}). Where no MBC was achieved, significance was calculated based on highest tested concentration.
Red bars denote peptides did not achieve a MBC at the described concentration. No data is provided for Defb14-1C^{V}Δ(1-5) tested against *E.faecalis* **(c)** due to lack of peptide stocks.

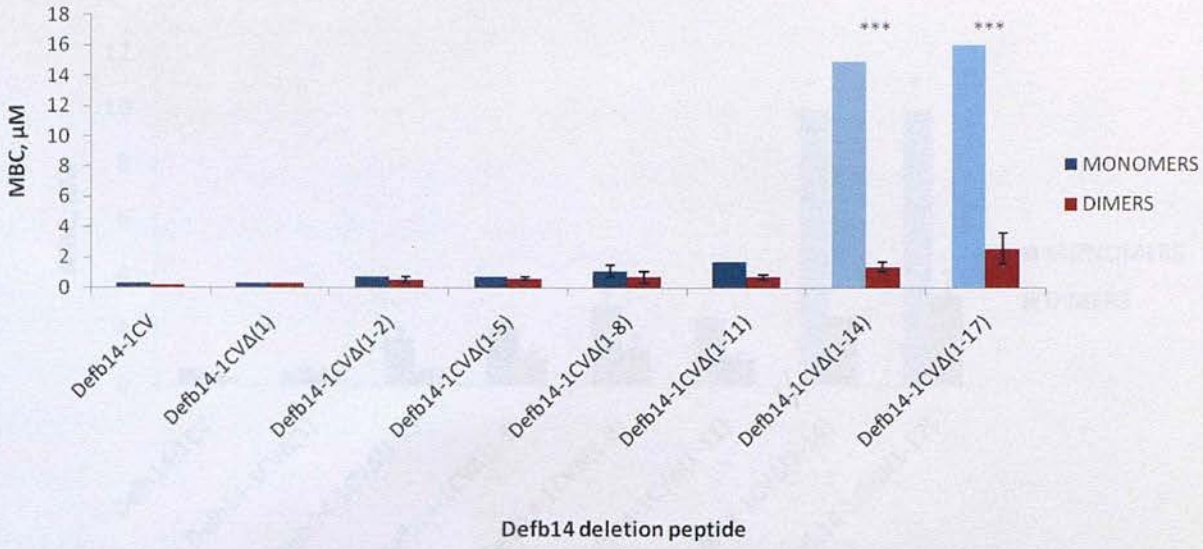


Figure 2.5 Comparison of the antimicrobial activity of monomeric and dimeric forms of the Defb14-1C^{V} deletion series against *P.aeruginosa*

This graph shows the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM). Bars faded to a lighter shade denote peptides that did not achieve an MBC at the depicted highest concentration tested (MBC > xµM). Values are based on figures for at least three independent experimental repeats. ***$p<0.0005$ (monomer vs. dimer). Where no MBC was achieved, significance was calculated based on highest tested concentration.

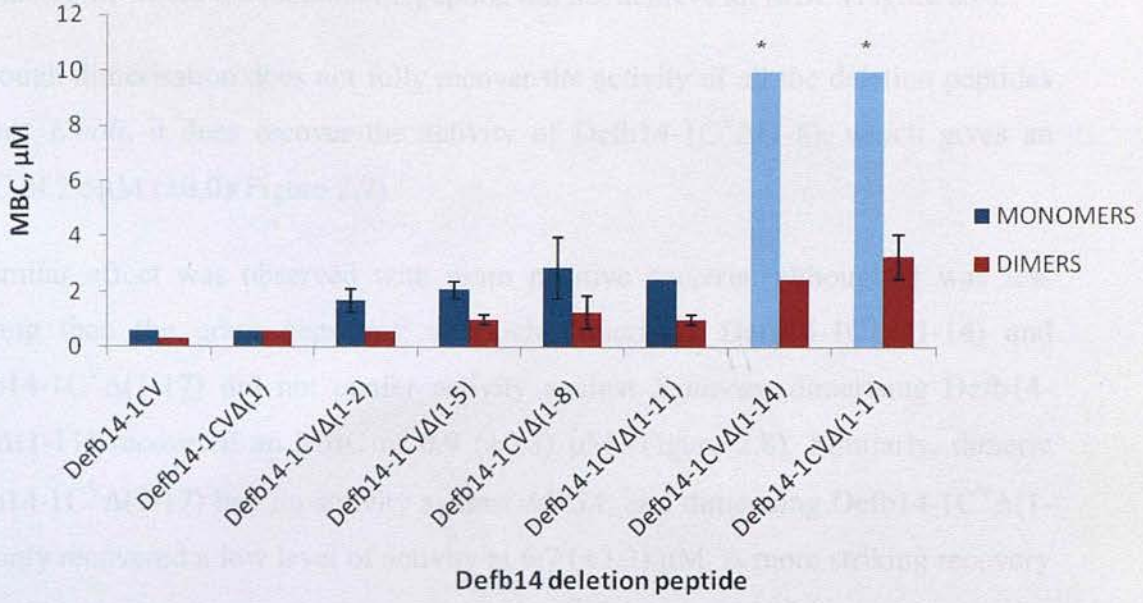


Figure 2.6 Comparison of the antimicrobial activity of monomeric and dimeric forms of the Defb14-1C^{V} deletion series against *A.baumannii*

This graph shows the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM). Bars faded to a lighter shade denote peptides that did not achieve an MBC at the depicted highest concentration tested (MBC > xµM). Values are based on figures for at least three independent experimental repeats.
*$p<0.05$ (monomer vs. dimer). Where no MBC was achieved, significance was calculated based on highest tested concentration.

dimer records an MBC of 2.6 (±1.0) μM, compared to an inactive monomer. Similarly, dimeric Defb14-1C^{V}Δ(1-17) has an MBC of 3.3 (±0.8) μM against *A.baumannii*, where the monomeric peptide did not achieve an MBC (Figure 2.6).

Although dimerisation does not fully recover the activity of all the deletion peptides against *E.coli*, it does recover the activity of Defb14-1C^{V}Δ(1-8), which gives an MBC of 2.5μM (±0.0)(Figure 2.7).

A similar effect was observed with gram positive bacteria, although it was less striking than the gram negatives; although dimerising Defb14-1C^{V}Δ(1-14) and Defb14-1C^{V}Δ(1-17) did not confer activity against *S.aureus*, dimerising Defb14-1C^{V}Δ(1-11) recovered an MBC of 0.9 (±0.3) μM (Figure 2.8). Similarly, dimeric Defb14-1C^{V}Δ(1-17) had no activity against *MRSA*, and dimerising Defb14-1C^{V}Δ(1-14) only recovered a low level of activity at 6.7 (±3.3) μM. A more striking recovery of activity occurred upon dimerising Defb14-1C^{V}Δ(1-11), which recovered an MBC of 1.5 (±1.1) μM against *MRSA* (Figure 2.9). Dimerisation had no effect on the lack of antimicrobial activity of Defb14-1C^{V}Δ(1-11), Defb14-1C^{V}Δ(1-14) and Defb14-1C^{V}Δ(1-17) against *E.faecalis*, however dimerising Defb14-1C^{V}Δ(1-2) and Defb14-1C^{V}Δ(1-8) resulted in a significantly improved ($p<0.05$) antimicrobial activity, with MBCs of 1.0 (±0.2) μM and 1.9 (±0.6) μM being recorded respectively (Figure 2.10). A summary of the Defb14-1CV deletion series MBC values for both monomers and dimers is shown in Table 2.3.

2.3.3 N-terminal substitutions have no effect on peptide activity

Previous work has shown that the loss of just two N-terminal amino acids abolishes Defb14 chemoattraction of CD4+ T cells (Tyrrell *et al.*, 2009). This study also showed that the mutation of the highly conserved leucine residue at position 2 (to glycine, lysine and isoleucine) resulted in differential activity changes, suggesting that this residue is crucial for chemotactic ability. It was therefore decided to investigate whether these changes would also affect the bactericidal activity of the parent peptide.

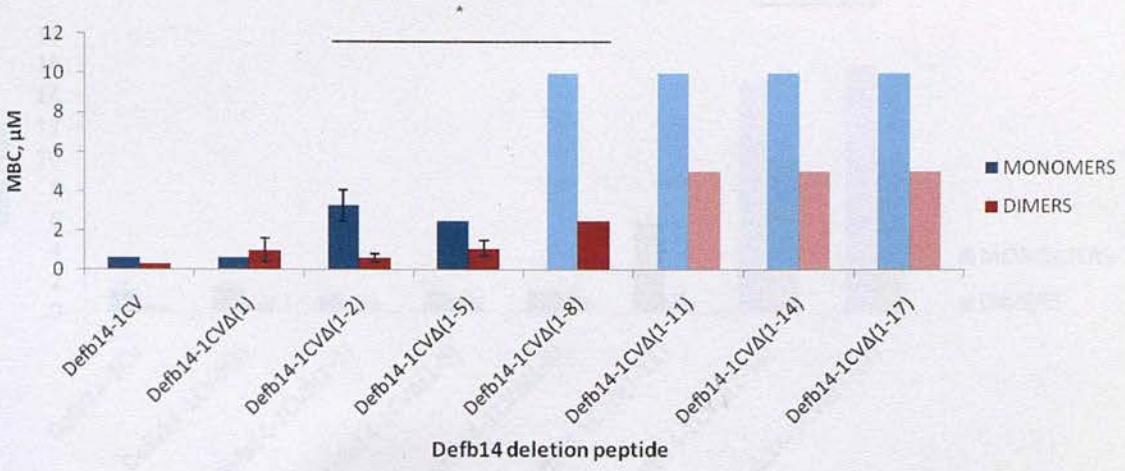


Figure 2.7 Comparison of the antimicrobial activity of monomeric and dimeric forms of the Defb14-1C^{V} deletion series against *E.coli*

This graph shows the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM). Bars faded to a lighter shade denote peptides that did not achieve an MBC at the depicted highest concentration tested (MBC > xμM). Values are based on figures for at least three independent experimental repeats.
*$p<0.05$ (monomer vs. dimer). Where no MBC was achieved, significance was calculated based on highest tested concentration.

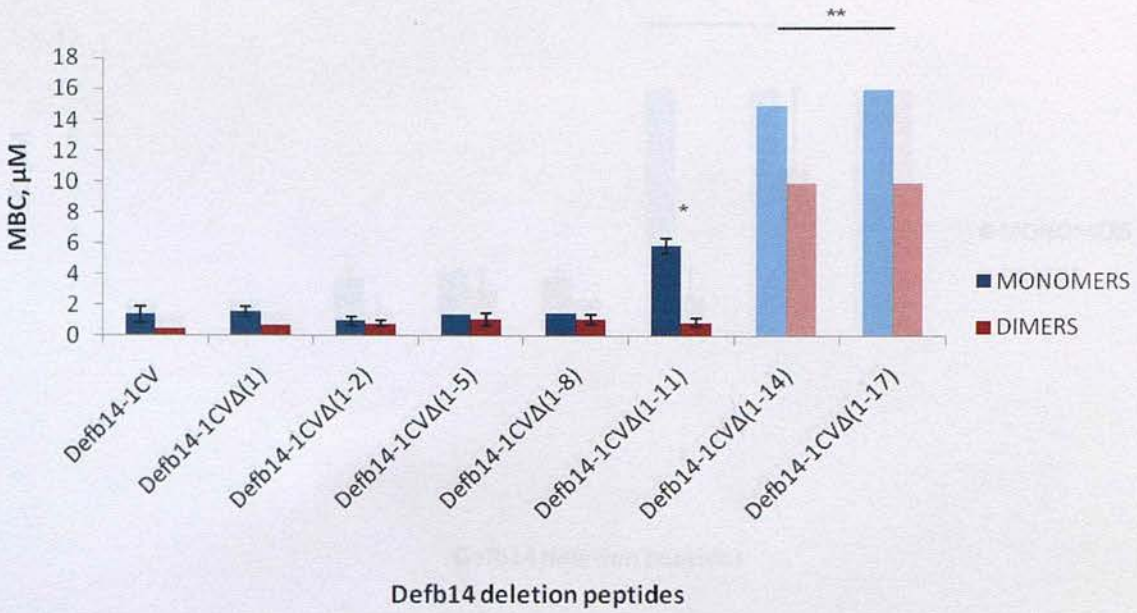


Figure 2.8 Comparison of the antimicrobial activity of monomeric and dimeric forms of the Defb14-1C^{V} deletion series against *S.aureus*

This graph shows the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM). Bars faded to a lighter shade denote peptides that did not achieve an MBC at the depicted highest concentration tested (MBC > xμM). Values are based on figures for at least three independent experimental repeats. *$p<0.025$, **$p<0.0001$ (monomer vs. dimer). Where no MBC was achieved, significance was calculated based on highest tested concentration.

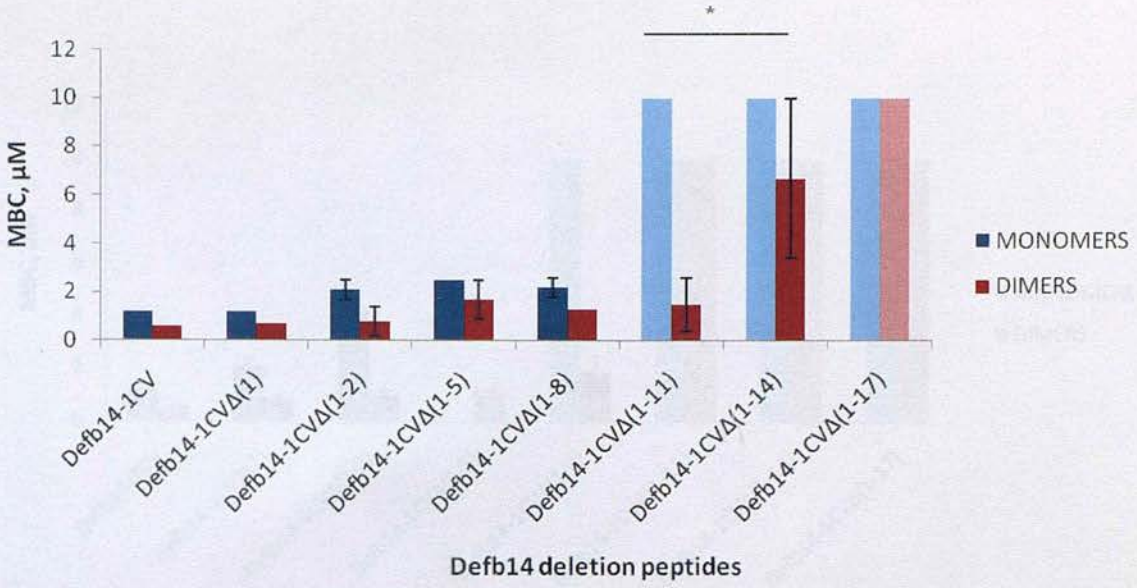


Figure 2.9 Comparison of the antimicrobial activity of monomeric and dimeric forms of the Defb14-1C^{V} deletion series against *MRSA*

This graph shows the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM). Bars faded to a lighter shade denote peptides that did not achieve an MBC at the depicted highest concentration tested (MBC > xµM). Values are based on figures for at least three independent experimental repeats.
*$p<0.05$ (monomer vs. dimer). Where no MBC was achieved, significance was calculated based on highest tested concentration.

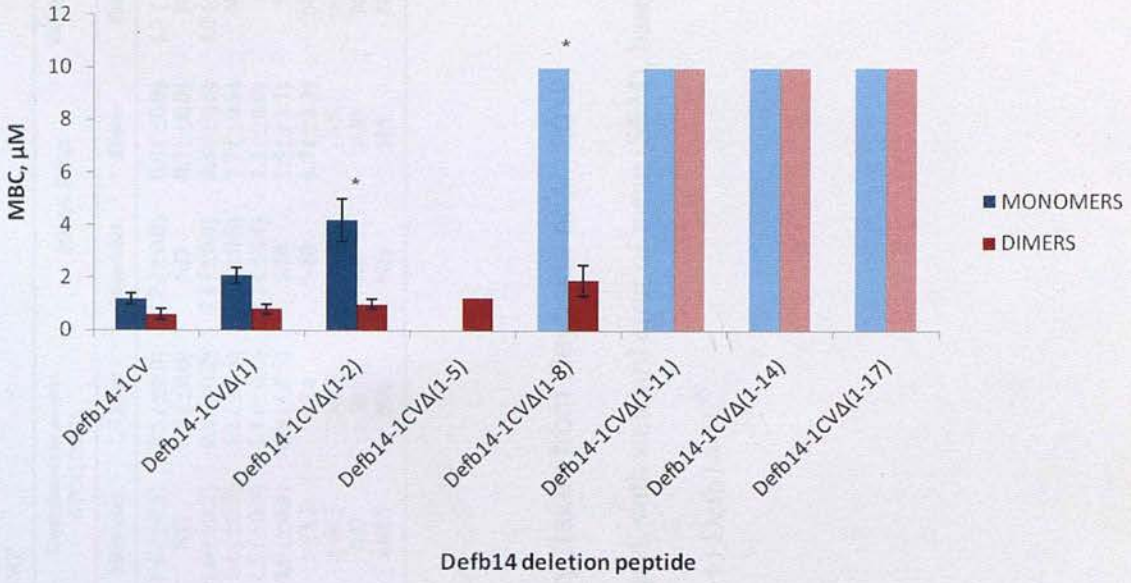


Figure 2.10 Comparison of the antimicrobial activity of monomeric and dimeric forms of the Defb14-1C^{V} deletion series against *E.faecalis*

This graph shows the minimum bactericidal concentration (MBC) achieved for each peptide ± standard error of mean (SEM). Bars faded to a lighter shade denote peptides that did not achieve an MBC at the depicted highest concentration tested (MBC > xµM). Values are based on figures for at least three independent experimental repeats.
*$p<0.05$ (monomer vs. dimer). Where no MBC was achieved, significance was calculated based on highest tested concentration.

Peptide	MBC (μM)[a]											
	Pseudomonas aeruginosa PAO1		*Escherichia coli* ATCC 25922		*Acinetobacter* baumannii ATCC 19606		*Staphylococcus aureus* ATCC 25923		MRSA J2918		Enterococcus faecalis ATCC 700802	
	Monomer	Dimer	Monomer	Dimer	Monomer	Dimer	Monomer	Dimer	Monomer	Dimer	Dimer	Monomer
Defb14-1C^{V}	0.3 (±0.0)	0.2 (±0.0)	0.6 (±0.0)	0.3 (±0.0)	0.6 (±0.0)	0.3 (±0.0)	1.4 (±0.5)	0.5 (±0.0)	1.2 (±0.0)	0.6 (±0.0)	1.2 (±0.2)	0.6 (±0.2)
Defb14-1C^{V}Δ(1)	ND	0.3 (±0.0)	ND	1.0 (±0.6)	ND	0.6 (±0.0)	ND	0.7 (±0.0)	ND	0.7 (±0.0)	ND	0.8 (±0.2)
Defb14-1C^{V}Δ(1-2)	0.7 (±0.0)	0.5 (±0.2)	3.3 (±0.8)	0.6 (±0.2)	1.7 (±0.4)	0.6 (±0.0)	1.0 (±0.3)	0.8 (±0.2)	2.1 (±0.4)	0.8 (±0.6)	4.2 (±0.8)	1.0 (±0.2)
Defb14-1C^{V}Δ(1-5)	0.7 (±0.0)	0.6 (±0.1)	2.5 (±0.0)	1.1 (±0.4)	ND	1.0 (±0.2)	1.4 (±0.0)	1.1 (±0.4)	2.5 (±0.0)	1.7 (±0.8)	ND	1.25 (±0.0)
Defb14-1C^{V}Δ(1-8)	1.1 (±0.4)	0.7 (±0.4)	**10 (±0.0)**	2.5 (±0.0)	2.9 (±1.1)	1.3 (±0.6)	1.5 (±0.0)	1.1 (±0.3)	2.2 (±0.4)	1.3 (±0.0)	**>5**	1.9 (±0.6)
Defb14-1C^{V}Δ(1-11)	1.7 (±0.0)	0.7 (±0.2)	>10	>5	2.5 (±0.0)	1.0 (±0.2)	**5.9 (±0.5)**	0.9 (±0.3)	**>10**	1.5 (±1.1)	>5	>5
Defb14-1C^{V}Δ(1-14)	**>15.2**	1.4 (±0.3)	>10	>5	**>10**	2.5 (±0.0)	>15.2	>7.6	>10	6.7 (±3.3)	>10	>5
Defb14-1C^{V}Δ(1-17)	>16.5	2.6 (±1.0)	>10	>5	>10	3.3 (±0.8)	>16.5	>8.3	>10	>5	>10	>5
Defb14-1C^{V}Δ(1-23)	ND	>10	ND	>10	ND	>10	ND	>10	ND	>10	ND	>10
Defb14-1C^{V}(18-23)	>83.5	NA	ND	ND	ND	ND	>83.5	NA	ND	NA	ND	ND

Table 2.3 Summary of Defb14-1C^{V} deletion series antimicrobial activity (taken from Reynolds *et al.*, 2010)

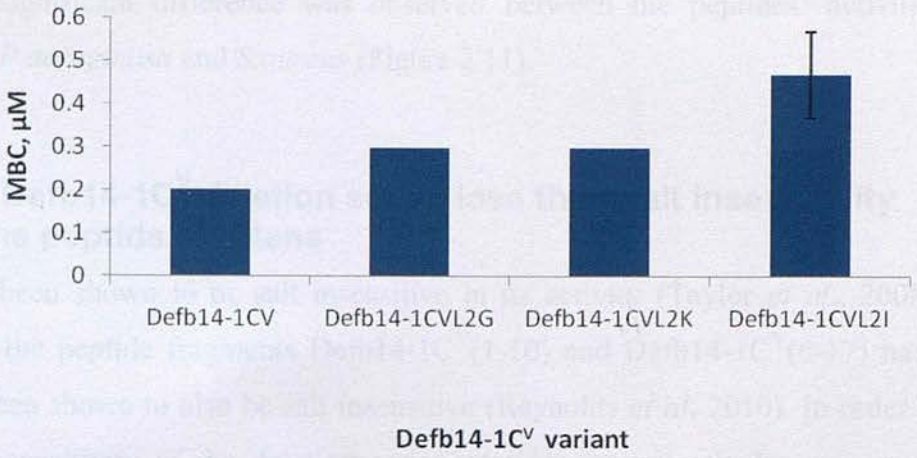
This table summarises the findings shown in figures 2.3-2.10. MBCs are given in μM, with standard error of mean (SEM), based on at least three replicate analyses.
Values in **bold** represent the first instance of statistical significance in comparison to Defb14-1C^{V}
ND = not done
NA = not applicable

Peptide	Sequence
Defb14-1C^{V}	F**L**PKTLRKFF**C**RIRGGR**C**AVLN**C**LGKEEQIGR**C**SNSGRK**CC**RKKK
Defb14-1C^{V}(L2G)	FGPKTLRKFF**C**RIRGGR**C**AVLN**C**LGKEEQIGR**C**SNSGRK**CC**RKKK
Defb14-1C^{V}(L2I)	FIPKTLRKFF**C**RIRGGR**C**AVLN**C**LGKEEQIGR**C**SNSGRK**CC**RKKK
Defb14-1C^{V}(L2K)	FKPKTLRKFF**C**RIRGGR**C**AVLN**C**LGKEEQIGR**C**SNSGRK**CC**RKKK

Table 2.4 Sequences of the Defb14-1C^{V} substitution peptides

Substituted peptides at position 2 are depicted in **red**. Defb14-1C^{V}(L2G) and Defb14-1C^{V}(L2K) showed poor chemotactic activity in comparison to the parent peptide (Tyrrell *et al.*, 2009).

a



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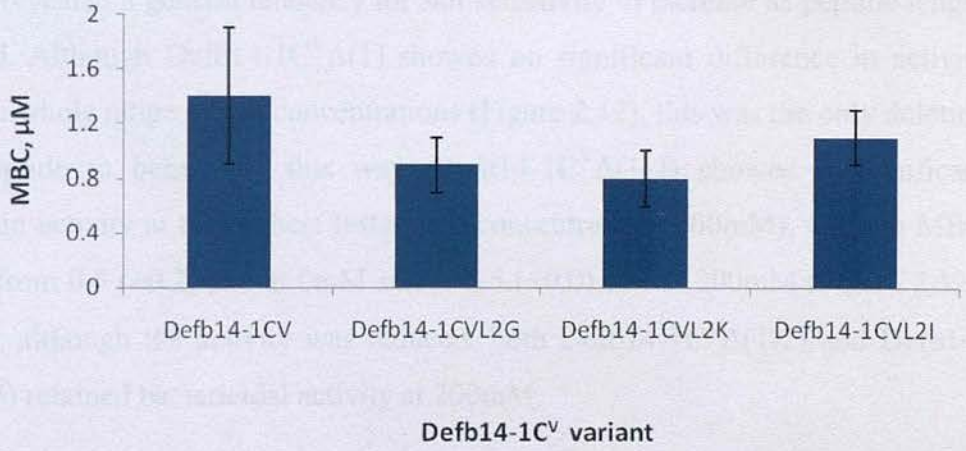


Figure 2.11 Antimicrobial activity of three Defb14-1C^{V} peptides with substitutions at position 2

(a) This graph depicts antimicrobial activity against gram negative *P.aeruginosa*. Data is based on three replicate experiments and error bars depict the standard error of the mean

(b) This graph depicts antimicrobial activity of the peptides against gram positive *S.aureus*.

None of the peptides showed a statistical difference in activity in comparison to the parent Defb14-1C^{V} peptide.

The three Defb14-1C^{V} substitution peptides (Table 2.4) were tested for antimicrobial activity against *P.aeruginosa* as a representative gram negative species and *S.aureus* as a representative gram positive species. It was found that in their dimeric state, no statistically significant difference was observed between the peptides' activities against both *P.aeruginosa* and *S.aureus* (Figure 2.11).

2.3.4 The Defb14-1C^{V} deletion series lose their salt insensitivity as the peptide shortens

Defb14 has been shown to be salt insensitive in its activity (Taylor *et al.*, 2008). Importantly, the peptide fragments Defb14-1C^{V}(1-10) and Defb14-1C^{V}(6-17) have previously been shown to also be salt insensitive (Reynolds *et al*, 2010). In order to test the salt sensitivity of the deletion series peptides, antimicrobial assays were performed against *P.aeruginosa* in the presence of increasing concentrations of sodium chloride salt, ranging from 0-200mM.

Analysis revealed a general tendency for salt sensitivity to increase as peptide length decreased. Although Defb14-1C^{V}Δ(1) showed no significant difference in activity across the whole range of salt concentrations (Figure 2.12), this was the only deletion series peptide to behave in this way. Defb14-1C^{V}Δ(1-2) showed a significant decrease in activity at the highest tested salt concentration (200mM), with an MBC increase from 0.5 (±0.2) μM at 0mM salt to 2.5 (±0.0) μM at 200mM (Figure 2.12). However, although the activity was reduced, both Defb14-1C^{V}Δ(1-2) and Defb14-1C^{V}Δ(1-5) retained bactericidal activity at 200mM.

A deletion of more than five amino acids proved to have a greater effect on bactericidal activity; Defb14-1C^{V}Δ(1-8) showed a significantly lower MBC of 6.7 (±2.0) μM ($p<0.05$) at only 50mM salt, with no activity at 100mM and 200mM. However, this trend toward inactivity appeared to level off at lower salt concentrations, with the largest deletion peptide Defb141C^{V}Δ(1-17) still maintaining a low level of bactericidal activity (MBC = 10±0.0μM) at 50mM salt (Figure 2.12).

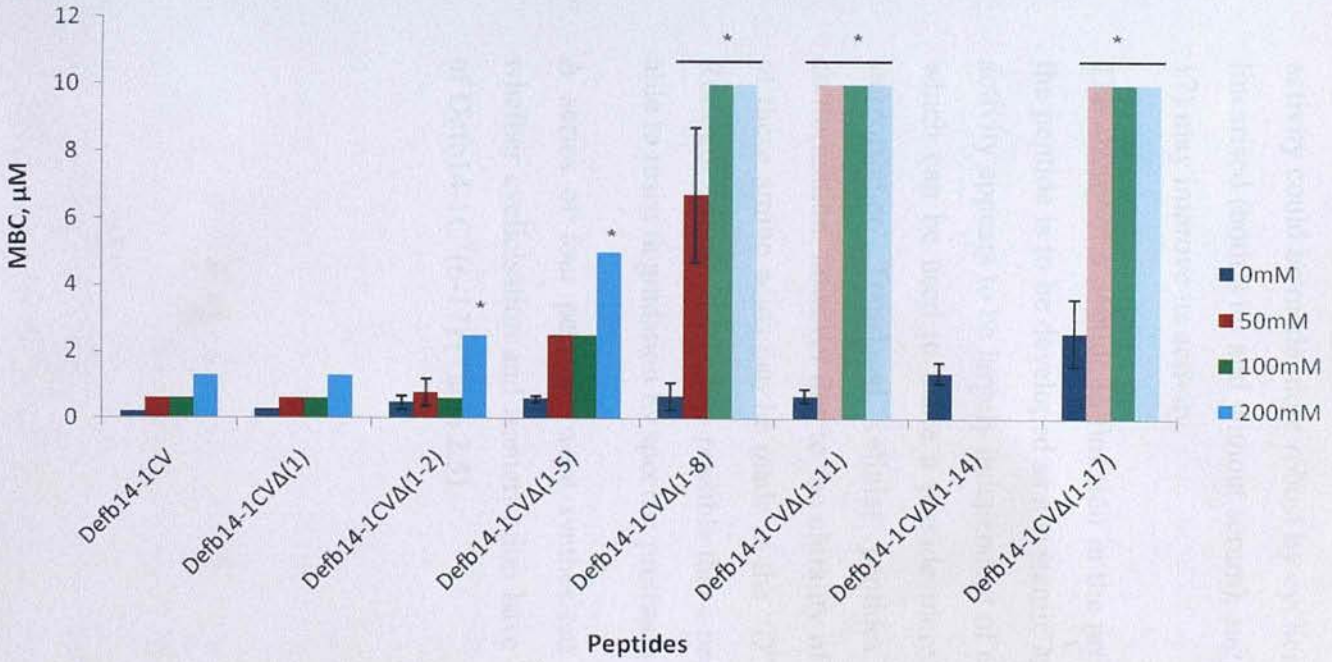


Figure 2.12 Effects of salt on the antimicrobial activity of the Defb14-1C^{V} deletion series

This graph depicts the antimicrobial activity of the dimeric Defb14-1C^{V} deletions series against gram negative *P.aeruginosa* in four different NaCl salt concentrations. Data is based on three independent repeats and the error bars represent the standard error of mean (SEM). Bars faded to a lighter shade (Defb14-1C^{V}Δ(1-8), Defb14-1C^{V}Δ(1-11) and Defb14-1C^{V}Δ(1-17)) represent peptides which did not achieve an MBC at the depicted concentration.

*$p<0.05$ with respect to 0mM peptide value. Where no MBC was achieved, significance was calculated based on highest tested concentration.

2.3.5 Improving the antimicrobial activity of Defb141C^{V}(6-17): serum sensitivity, cyclicisation and stereoisomers

Since Defb14-1C^{V}(6-17) had previously been identified as a short antimicrobially active fragment of Defb14-1C^{V} (Reynolds *et al.*, 2010), I decided to investigate the activity of this peptide in the presence of serum and see whether the antimicrobial activity could be made more robust by cyclicisation. Tang *et al.*, 1999 suggested that linearised (both with and without serum), suggesting that cyclicising Defb14-1C^{V}(6-17) may improve its activity.

The ability of a peptide to function in the presence of serum is clinically relevant if the peptide is to be developed as a systemic antibiotic. However, since antimicrobial activity appears to be largely independent of exact 3D peptide structure, one strategy which can be used to make a peptide more stable *in vivo* is the production of a stereoisomer. Translated cellular peptides contain amino acids in the "L" conformation, however due to the chirality of carbon, stereoisomer "mirror images" of these amino acids can be made in the "D" conformation (reviewed in Martínez-Rodríguez *et al.*, 2010). It is possible that a peptide made of D-amino acids would be able to resist degradation by specific proteases better than the L-form.

A series of four peptides were synthesised (by Derek Macmillan, UCL) to test whether cyclicisation and isomerisation have an effect on the antimicrobial activity of Defb14-1C^{V}(6-17) (Table 2.5).

Peptide	Sequence
Defb14-1C^{V}(6-17)	LRKFFARIRGGR
Defb14-1C^{V}(7-17)^{LIN}	CRKFFARIRGGR
Defb14-1C^{V}(7-17)^{CYC}	CRKFFARIRGGR
[Defb14-1C^{V}(7-17)^{LIN}]D	CRKFFARIRGGR
[Defb14-1C^{V}(7-17)^{CYC}]D	CRKFFARIRGGR

Table 2.5 The sequences of Defb14-1C^{V}(6-17) derivatives

This table shows the sequences of four synthetic derivatives of Defb14-1C^{V}(6-17) in comparison to the original Defb14-1C^{V}(6-17). A cysteine residue was added to the N-terminal of the peptides to facilitate cyclicisation. The superscript "^{LIN}" refers to a linear molecule and "^{CYC}" denotes a cyclic peptide. The final two peptides, followed by the letter "D" denote stereoisomers, containing only D-amino acids.

A new preparation of Defb14-1C^{V}(6-17) was tested for antimicrobial activity against *P.aeruginosa* as a representative gram negative organism, where it achieved a MBC of 1.6 (±0.3) μM. However, when tested in 5% (v/v) foetal calf serum (FCS) antimicrobial activity was abrogated (MBC > 20μM). A similar observation was made with the Defb14-1C^{V}(7-17)^{LIN} peptide, which achieved a MBC of 2.4 (±0.1) μM in 0% serum, but lost activity (MBC > 18μM) in just 5% (v/v) serum. The cyclicisation of the Defb14-1C^{V}(7-17)^{CYC} peptide did not improve the antimicrobial activity in either serum-free or 5% serum (Figure 2.13).

Instead, the peptide showed lowered antimicrobial activity in serum-free tests (MBC=3.6±0.7μM) and lost activity in 5% serum (MBC > 19μM).

Interestingly, although the Defb14-1C^{V}(7-17)^{LIN}D peptide did not display antimicrobial activity as potent as Defb14-1C^{V}(6-17), the activity appeared to be more robust. Figure 2.13 shows that Defb14-1C^{V}(7-17)^{LIN}D maintains antimicrobial activity even in 10% FCS, although the concentration of peptide required to kill 99.99% inoculum is significantly increased. The Defb14-1C^{V}(7-17)^{CYC}D peptide shows a similar level of activity to Defb14-1C^{V}(7-17)^{LIN}D, although the MBC

achieved at 10% FCS was not significantly different to that achieved at 0% FCS (6.9 ± 1.6μM vs 10.0 ± 0.0μM, respectively). This suggests that the cyclicisation of the D-amino acid stereoisomer may act to further preserve the antimicrobial activity of the linear peptide.

The analysis was repeated using *S.aureus* as a representative gram positive organism. As seen with the *P.aeruginosa* analysis, cyclicisation had no effect on improving the antimicrobial activity of Defb14-1C^{V}(7-17)^{LIN} either in serum-free or 5% FCS reactions (Figure 2.14). However, the D-amino acid isomerisation improved the Defb14-1C^{V}(7-17)^{LIN}D at all serum concentrations and a MBC was recorded even at the highest tested serum concentration (MBC = 20 ±0.0 μM). Critically, the

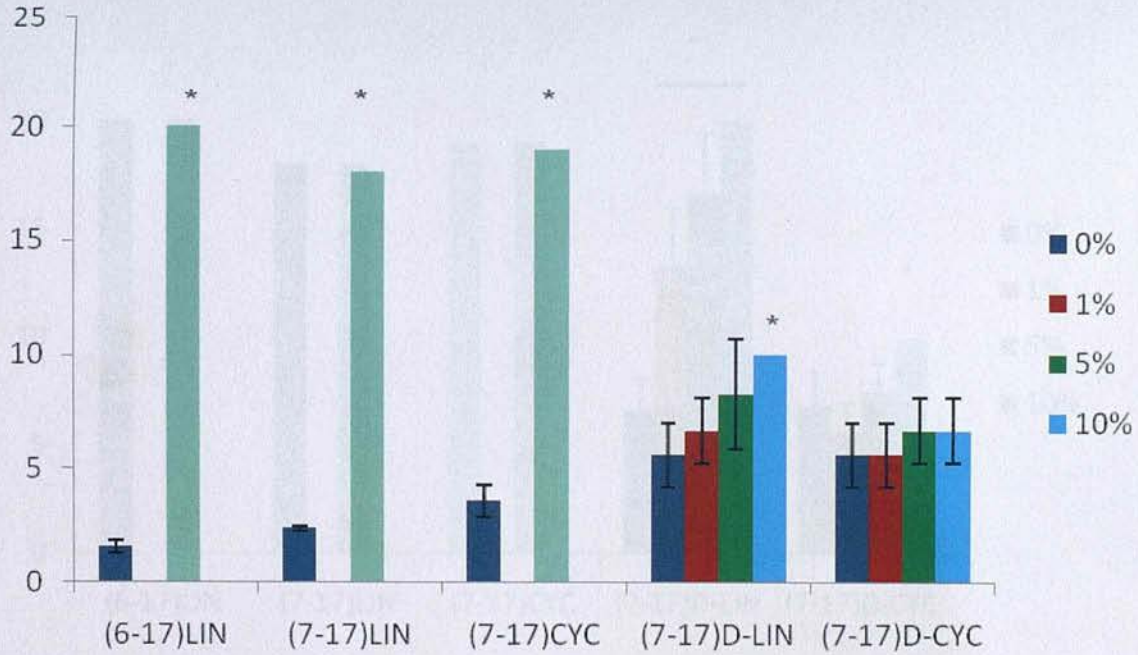


Figure 2.13 Serum sensitivity of Defb14-1C^{V}(6-17) derivatives against gram negative *P.aeruginosa*

Peptides were tested for antimicrobial activity against *P.aeruginosa* in the presence of increasing concentrations of foetal calf serum (FCS), ranging from 0% to 10% (v/v). Faded out bars denote peptides which did not achieve a MBC at the depicted concentration. *$p<0.05$ (vs peptide at 0% serum), and where a MBC was not achieved, significance was calculated using the maximum tested peptide concentration.
Data is based on three independent repeats and error bars denote standard error of mean (SEM).
Assays were not carried out in 1% and 10% serum for peptides (6-17)^{LIN}, (7-17)^{LIN} and (7-17)^{CYC}.

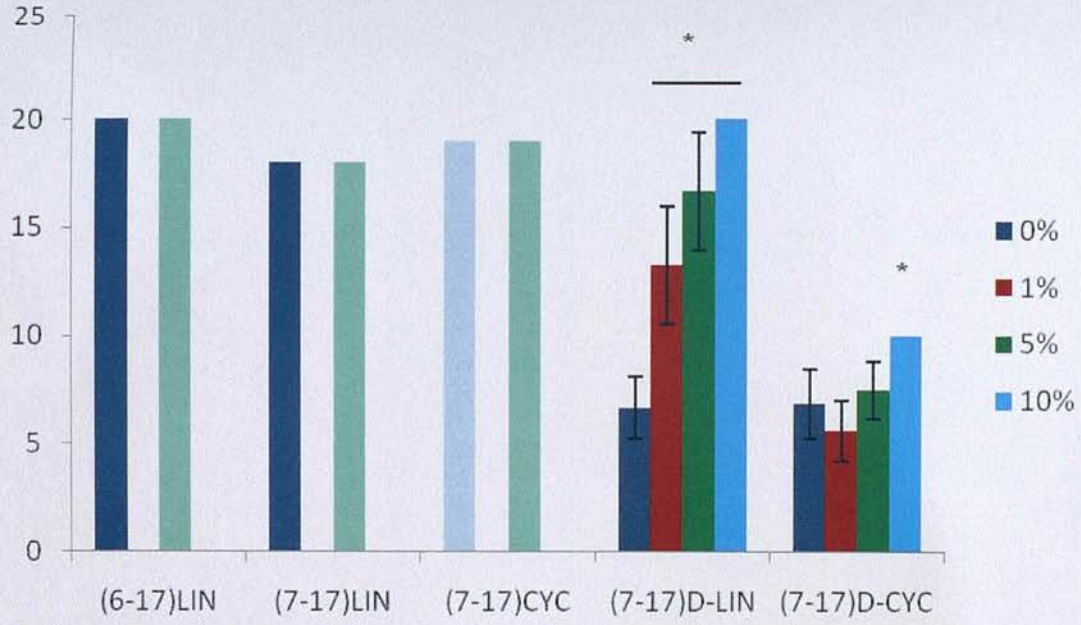


Figure 2.14 Serum sensitivity of Defb14-1C^{V}(6-17) derivatives against gram negative *S.aureus*

Peptides were tested for antimicrobial activity against *S.aureus* in the presence of increasing concentrations of foetal calf serum (FCS), ranging from 0% to 10% (v/v). Faded out bars denote peptides which did not achieve a MBC at the depicted concentration.
*$p<0.05$ (vs peptide at 0% serum), and where a MBC was not achieved, significance was calculated using the maximum tested peptide concentration.
Data is based on three independent repeats and error bars denote standard error of mean (SEM).
Assays were not carried out in 1% and 10% serum for peptides (6-17)^{LIN}, (7-17)^{LIN} and (7-17)^{CYC}.

isomerisation also appeared to improve the peptides activity in serum-free medium. The Defb14-1C^{V}(7-17)^{LIN} linear peptide achieves a MBC of 18 ±0.0μM in 0% serum, however Defb14-1C^{V}(7-17)^{LIN}D showed 99.99% kill of the initial inoculum at just 6.7±1.4μM. The cyclic isomer showed further improvement over the linear, with antimicrobial activity at 10% FCS increasing in this peptide to lead to a recorded MBC of 10 ± 0.0μM, nearly half the value of the linear peptide.

2.4 DISCUSSION

This work has continued the analysis of the Defb14 peptide with the ultimate aims of elucidating the origins of antimicrobial activity and assessing its suitability for development as a potential novel antibiotic. To approach the question of the importance of primary amino acid sequence to bactericidal activity, I tested a deletion library of Defb141C^{V}-derived peptides against a panel of clinically relevant bacterial strains. This deletion library has the collective power of illustrating the importance of fragments of peptide within the context of a full-length molecule, thus maintaining a conformation as close to the physiological parent peptide as possible.

I also investigated the effects of cyclicisation and isomerisation on antimicrobial activity within the context of a single Defb14-1C^{V} fragment, Defb14-1C^{V}(6-17).

2.4.1 Primary peptide sequence has a crucial role in the determination of bactericidal activity against different bacterial strains

In this work, I showed that as the Defb14-1C^{V} peptide deletions increased, that monomeric MBCs increased in a strain dependent manner. This analysis highlighted the importance of specific amino acid sequences to the bactericidal activity against different strains; based on the MBC values obtained, amino acids 6-8 (LRK) appear to be critical in monomeric activity against *E.coli* and *E.faecalis* (Figures 2.3c, 2.4c), amino acids 9-14 (FFARIR) in activity against *S.aureus* (Figure 2.4a) and amino acids 15-17 (RIR)in activity against *P.aeruginosa* and *A.baumannii* (Figures 2.3a,b).

This finding suggests that multiple sequences are important for the broad spectrum bactericidal activity seen in Defb14, but highlights in particular residues 6-17 as amino acids that are critical to the response against the organisms tested in this study. This is concordant with work previously carried out in this group, which showed that peptide fragments Defb14-1C^{V}(1-10) and Defb14-1C^{V}(6-17) display potent bactericidal activity (Reynolds *et al*, 2010). The increased activity of Defb14-1C^{V}(6-17) over Defb14-1C^{V}(1-10) is also supported by this data, since I have shown that residues 1-5 can be removed from the parent peptide without significant effects on the bactericidal activity. This indicates that the bactericidal activity of Defb14-1C^{V}(1-10) may derive mainly from amino acids 6-10 (LRKFF). However, the data

would also support amino acids 1-17 having individual additive effects to the bactericidal activity.

The highlighting of amino acids 6-8 as important in bactericidal activity (against *E.coli* and *E.faecalis*) is also concordant with a previous study on HBD3, which showed that the sequence NTLQK has been subject to negative selection forces throughout primate evolution (Semple *et al*, 2005). The sequence NTLQK corresponds to KTLRK in Defb14 (Figure 2.1), and its conservation in primates might be indicative of its importance in bactericidal activity.

These observations indicate that primary sequence is important in determining bactericidal activity, but do not differentiate between sequence and the overall molecular charge and hydrophobicity. Hydrophobicity is a measure of the proportion of hydrophobic residues present in a peptide and a higher hydrophobicity (less negative ΔG value) is thought to confer a greater ability for membrane integration and disruption.

Through their structure-function work on HBD3, Klüver *et al.*, 2005, argued that the most important factors for maintaining bactericidal activity were a combination of high positive charge and hydrophobicity, where decreases in charge could be compensated for by increases in hydrophobicity (Klüver *et al*, 2005). While, the work described in this chapter suggests that primary sequence affects activity independently of these factors, it also supports these claims. Figure 2.15 depicts the antimicrobial activity of deletion series against *P.aeruginosa* graphically, showing a progressive reduction of charge as the peptide deletions increase. The monomeric peptides Defb14-1C$^{V}\Delta$(1-17) and Defb14-1C^{V}(6-17) have the same charge, yet only

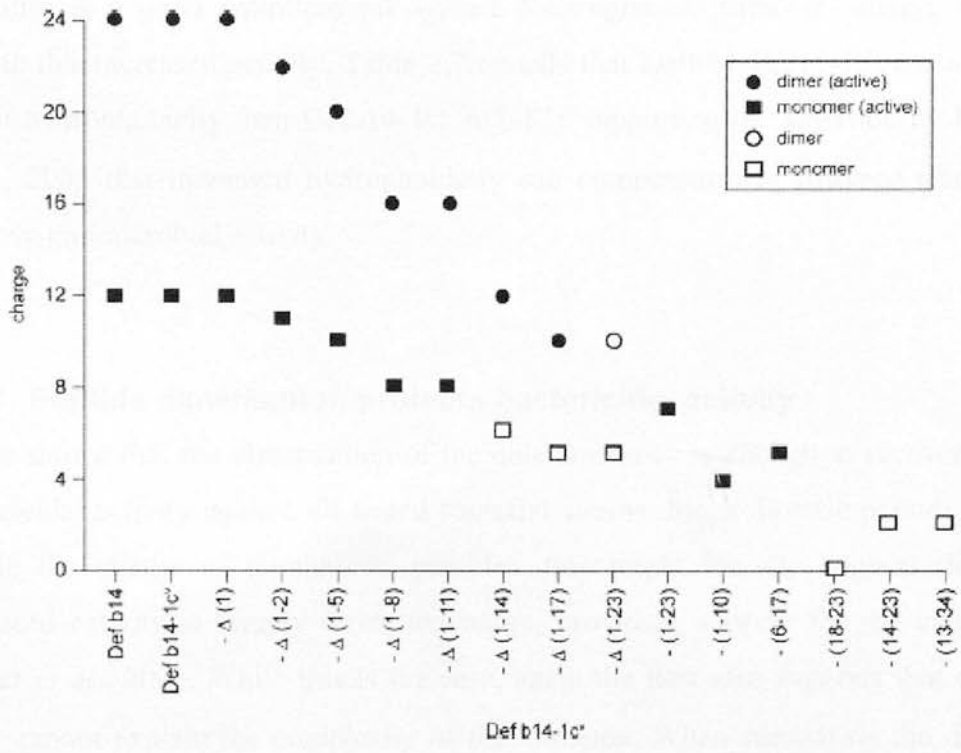


Figure 2.15 The relationship between charge and antimicrobial activity (taken from Reynolds *et al.*, 2010)

The above graph depicts the net charge (pH 7) of the Defb14-1C^{V} deletion series monomers and dimers. Monomers are denoted by a square, dimers by a circle. Active peptides are marked with black shading.

the latter is a good antimicrobial against *P.aeruginosa*. Although charge cannot explain this increased activity, Table 2.2 reveals that Defb14-1C^{V}(6-17) has a much higher hydrophobicity than Defb14-1C^{V}Δ(1-17), supporting the assertion by Klüver *et al.*, 2005 that increased hydrophobicity can compensate for lowered charge to improve antimicrobial activity.

2.4.2 Peptide dimerisation protects bactericidal activity

I have shown that the dimerisation of the deletion series is enough to recover some bactericidal activity against all tested bacterial strains. Since dimeric peptides have double the charge of monomeric peptides, this might initially suggest that the increased activity is largely down to charge, providing support for the claims of Klüver *et al.*, 2005. While this is the case, again the data also suggests that charge alone cannot explain the complexity of the situation. When comparing the dimeric forms of Defb14-1C^{V}Δ(1-17) and Defb14-1C^{V}Δ(1-23) in Figure 2.15, it can be seen that the peptides possess the same charge. However, while both peptides are inactive against *P.aeruginosa* in their monomeric forms, Defb14-1C^{V}Δ(1-17) alone acquires activity as a dimer (Figure 2.5). Since the peptides possess similar hydrophobicities, it would be reasonable to expect Defb14-1C^{V}Δ(1-23) to behave in a similar way, if charge and hydrophobicity were the only considerations to be made. Since the peptides' activities are so vastly different, it leads to the conclusion that the addition of amino acids AAVLNA in Defb14-1C^{V}(1-17) confer additional bactericidal activity. Interestingly, the peptide AAVLNA is inactive (and unable to form dimers)(data not shown), so the extra sequence is only relevant within the context of the larger molecule. This finding supports our experimental technique of investigating the activity of amino acid sequences within the context of a larger parent peptide.

The dimerisation data described in this chapter is not the first observation of its kind. It supports the recent work of Antcheva *et al.*, 2009, who conducted a study on an artificial β-defensin, synthesised based on the shared characteristics of more than 80 defensins. Their work suggested that dimerisation could improve bactericidal activity of the peptide, although no comment was made about the influence of molecular

charge on this activity (Antcheva *et al.*, 2009). Neither the findings of Antcheva *et al.*, 2009 nor the findings reported in this chapter explain why Defb14-1C is no more active than Defb14, despite being a dimer. It perhaps suggests that there is a ceiling to the bacterial killing efficiency a peptide can have, or otherwise it may indicate that activity is being limited by another independent factor.

2.4.3 Salt sensitivity increases as the N-terminal deletions of Defb14-1C^{V} increase

Defb14 and Defb14-1C^{V} are salt insensitive peptides and maintain a high level of antimicrobial activity at high (200mM) concentrations. The salt concentration in physiological isotonic saline solutions is approximately 150mM (0.9% NaCl), so maintaining antimicrobial activity at these levels is crucial for any peptide which may be developed for future use as an intravenous medicine. In addition, Goldman *et al.*, 1997 found that fluid from the airways of cystic fibrosis patients contained salt at a concentration of 120mM. Resistance to high levels of salt may allow for the development of an antibiotic inhaler to treat cystic fibrosis patients.

Figure 2.12 shows that at 200mM NaCl, a significant decrease in Defb14-1C^{V} antimicrobial activity against *P.aeruginosa* is observed following the deletion of just two N-terminal amino acids. Moreover, the removal of the first eight N-terminal amino acids abrogates antimicrobial activity at this salt concentration. This data suggests that the N-terminal region is highly important for the retention of salt insensitivity and that this property is more sensitive than antimicrobial killing in salt-free solutions.

It is possible that the N-terminal confers additional stability to Defb14-1C^{V} under high salt conditions and is thus required for antimicrobial activity. Alternatively, since the mechanism of defensin activity is thought to derive from initial electrostatic interactions with the negatively charged bacterial cell wall, it could be that the presence of Na^{+} cations disrupts this electrostatic balance. Since the Defb14-1C^{V} deletion series progressively decreases in charge (Figure 2.15), this might explain the observed effects.

2.4.4 The serum sensitivity of Defb14-1C^{V}(6-17) derivatives can be manipulated by chiral isomerisation

In this chapter, I showed that the chiral isomerisation of Defb14-1C^{V}(7-17) by substitution of L-amino acids for D-amino acids results in a marked increase in antimicrobial performance in the presence of serum. This improvement was observed in both gram positive and negative bacteria and resulted in resistance in up to 10% serum (Figures 2.13, 2.14). In addition, cyclicisation of the chiral isomer further improved the antimicrobial activity, in particular against gram positive *S.aureus*.

Since the majority of cellular proteins are synthesised using L-amino acids, it is possible that D-form peptides are resistant to proteolytic degradation *in vivo*, if they can no longer be recognised enzymatically. Similarly, even a reduction in proteolysis might result in an improvement in antimicrobial activity. Although the stability of the peptides were not tested in this study, this could be carried out using mass spectrometry in future work.

Tang *et al.*, 1999 postulated that the θ-defensin RTD-1 maintained a higher level of antimicrobial activity due to its cyclic nature. The findings in this chapter do not suggest that the same applies for Defb14-1C^{V}(7-17)^{CYC}. While this observation might be simply explained by the artificial nature of the tested peptide, there are a number of other explanations.

The lack of correlation to Tang *et al.*, 1999 might be explained by the difference in peptide sizes. Since RTD-1 is a full length 18 amino acid peptide, when Defb14-1C^{V}(7-17)^{CYC} is simply a 10 amino acid fragment of a much larger peptide, it may have reduced overall stability and therefore lowered antimicrobial activity. Alternatively, it may be that the cyclicisation of Defb14-1C^{V}(7-17)^{CYC} alters the 3D structure of the peptide too much for it to maintain its antimicrobial activity. In Figures 2.13 and 2.14 it is apparent that the cyclic peptides do not function as well as the linear in serum-free solutions, which may indicate a partial steric shielding of positively charged residues, critical for microbial membrane interactions.

While the cyclicisation of Defb14-1C^{V}(7-17) did not appear to dramatically improve the antimicrobial activity of the peptide, creating chiral D-form isomers did. This presents an interesting avenue for future research and indicates that when designing future antibiotics, D-form isomers should be considered and tested, to ensure maximum drug efficiency. However, since D-amino acids are found naturally in the peptidoglycan of bacterial cell walls (reviewed in Cava *et al.*, 2011), extra care would need to be taken to first determine whether the peptides would induce an immune response.

2.5 CONCLUSIONS AND FUTURE DIRECTIONS

In this chapter I have shown that as the Defb14-1C^{V} peptide truncates from the N-terminal, its MBC increases, indicating a poorer bactericidal activity. However, while this general trend was seen in all tested strains, the number of amino acids that can be lost before the peptide becomes inactive varies between organisms. Interestingly, the bactericidal activity against gram negative species appeared to be more robust than that of the gram positives.

In this work, I also showed that dimerisation improves the activity of deletion peptides and that changes in charge and hydrophobicity are insufficient to fully account for these large changes in activity.

Finally, I evaluated a series of cyclic and stereoisomeric peptides based on the antimicrobially active fragment Defb14-1C^{V}(6-17). I found that while cyclicising peptide fragments has a limited effect on the antimicrobial activity, creating stereoisomers using D-amino acids appears to confer an increased activity in the presence of serum (compared to L-form peptides). However, this increased activity was only observed in the presence of serum and not in serum-free reactions. This finding is especially relevant in the light of a recent study by Maisetta *et al.*, 2011 which showed that *P.gingivalis* can produce proteases which help resist antimicrobial peptide function. The finding that the D-form cyclic peptide tested in this study is resistant to serum, suggests the peptide is resistant to specific proteases, which may be important in tackling bacteria like *P.gingivalis*.

This study is interesting from two points of view. Firstly, it adds to the growing literature on the origins of antimicrobial activity in the β-defensins, and secondly, it has implications for the creation and modification of defensin-based antibiotics. However, this is not a fully comprehensive study and indicates a number of areas in which additional work could be carried out. A selection of these studies is suggested below.

A major limitation of this study is that it has been carried out using synthetic, modified peptides in an *in vitro* setting. Since the study was carried out on murine peptides, it would be interesting to mutagenise Defb14 and carry out *in vivo* studies

to determine whether my *in vitro* observations are replicated. In particular, it would be interesting to observe the effect of deleting triplet peptide sequences within the Defb14 molecule, to firstly observe any differences in disease clearance and susceptibility. Alternatively, primary mutant airways could be cultured and observed for differences in ability to fight controlled infections.

A second area of future work could be to investigate the amphiphilicity of the Defb14-1C^{V} deletion series. Although this study considered primarily the effect of charge and amino acid sequence on antimicrobial activity, work by Pathak *et al.*, 1995, suggested that amphiphilicity is the most important characteristic of an antimicrobial peptide (above hydrophobicity and helicity). Since this study only briefly considered the effects of hydrophobicity on antimicrobial activity, it may prove interesting to consider the changing amphiphilicity as the deletion series progresses. Amphiphilic peptides possess both hydrophobic and hydrophilic peptides, enabling them to interact with both charged and uncharged residues, Given the amphiphilic nature of cell membranes, this property is thought to allow β-defensins to efficiently insert into and disrupt membranes.

In order to further the interesting findings from the cyclic stereoisomer work described in this chapter, it would be of great value to carry out *in vivo* analysis of the Defb14-1C^{V}(7-17)^{CYC}D. Since the antimicrobial activity of this peptide was so promising *in vitro*, it would be interesting to observe whether administering the peptide to mice suffering from acute infection would alleviate the disease. Demonstrating that the peptide could work effectively *in vivo* would be an important step in identifying it as a potential novel antibiotic.

Finally, to further the *in vitro* analysis carried out in this study, it would be interesting to carry out a reciprocal study looking at the effects of deleting the C-terminal of Defb14-1C^{V}. Since the N-terminal of Defb14 seems to be the most important for antimicrobial activity, it would be interesting to take the active Defb14-1C^{V}(1-23) and progressively truncate it. This would identify whether the active sequences identified in this chapter are only active within the context of a full-length molecule or whether they are independently highly active peptides, suitable for development as novel antibiotics.

Chapter 3: The Antimicrobial Function of the Zebrafish Defensins

"I know the human being and fish can co-exist peacefully"

Presidential candidate George W. Bush

3.1 PREFACE

The zebrafish, *Danio rerio*, is a proven and clinically relevant organism on which to model immunological studies. In recent years, the study of infection and inflammation in zebrafish and other teleosts has become more widespread. These organisms have proved to be important in the study of the pathogenesis of bacterial infections, notably providing a relevant model for mycobacterial disease (Prouty *et al.*, 2003), as well as being useful in the study of viral infections, like the Herpes simplex virus (Burgos *et al.*, 2008).

As described in Chapter 1, antimicrobial peptides are important constituents of the mammalian innate immune system and directly provide protection against a range of microbes. The situation in aquatic species is less extensively studied than that of mammals, however there is growing evidence that antimicrobial peptides serve a similar purpose in a variety of fish and a recent review identified more than 80 antimicrobial peptides in various fish species (Rajanbabu and Chen, 2011). Some of the peptides do not have clear mammalian homologues; the antimicrobial peptide, pleurocidin (a piscidin), purified from skin mucal secretions of the winter flounder, *Pleuronectes americana* has been shown to have potent activity against both gram positive and negative species of bacteria (Cole, 1997). Similarly, other piscidins have been demonstrated to be potent haemolytic and antimicrobial peptides in the Perciformes teleosts (Silphaduang, 2001).

In addition, orthologues of the major categories of mammalian antimicrobial peptides have also been identified in fish and in the past five years, new genes have been rapidly identified. Notably, functional cathelicidins have been reported in the Rainbow Trout (*Oncorhynchus mykiss*), Atlantic Salmon (*Salmo salar*) (Chang *et al.*, 2006) and cod (*Gadus morhua*) (Broekman *et al.*, 2011) and defensin homologues have been identified in zebrafish (*Danio rerio*) and other teleosts (Zou *et al.*, 2007) and rainbow trout (Falco *et al.*, 2008; Casadei *et al.*, 2009). Recently, novel and antimicrobially active hepcidins have also been identified in orange-spotted grouper (*Epinephelus coioides*)(Zhou *et al.*, 2011).

As described in Chapter 1, three β-defensin-like peptides were reported by Zou *et al.*, 2007. Recently, Oehlers *et al.*, 2011 mapped the expression of these defensins across

the length of the intestine of both adult zebrafish and larvae, showing an increasing anterior-posterior gradient of expression. In this chapter, I describe the further characterisation of these β-defensin-like peptides, with a view to defining the role that these genes might play in the immunity of zebrafish as well as investigating their potential for development as novel antibiotics. The work includes a search for additional zebrafish β-defensins and observations on the expression of the genes in both embryos and adult fish. In addition, the antimicrobial properties of the peptides against a range of human and zebrafish pathogens are explored *in vitro*.

3.2 AIMS

The aims of this chapter were to:

1. Search the zebrafish genome for additional novel β-defensin-like genes
2. Investigate the expression and inducibility of these genes to form a better idea of their role in zebrafish immunity
3. Investigate the antimicrobial activity of the published zebrafish defensin-like peptides against a panel of microbes, including gram positive and negative bacteria and fungi
4. Investigate any antimicrobial activity under differing salt and serum concentrations to evaluate the potential for development as novel therapeutics

3.3 THE GENOMIC ORGANISATION OF THE ZEBRAFISH DEFENSINS

3.3.1 Searching for novel members of the zebrafish defensin family

Since the human β-defensins have undergone rapid gene duplication and diversification events throughout evolution (Semple *et al.*, 2005), it is reasonable to suppose that their zebrafish counterparts might have undergone the same processes. This supports the case for the presence of more than three defensin genes being present in the zebrafish genome. To address this, a comprehensive search of the genome was carried out, with the aim to further the initial discoveries reported in Zou *et al.*, 2007.

More than 100 β-defensin peptide sequences were collected from chicken, mouse, human and zebrafish using the Ensembl web server. These sequences were used to construct a Hidden Markoff Model which was then applied to the zebrafish genome using Genewise (with the assistance of Dr Colin Semple). Due to the large number of hits, this analysis was repeated (by Dr. Colin Semple), applying the model to the known zebrafish ESTs (Ensembl release 48, December 2007), to narrow the hits to genes that are expressed. This analysis produced 38 gene predictions, of which only 14 scored as highly as the known zebrafish defensins (which were also detected, providing a positive control for the analysis). Of the remaining 14 predictions, five were chosen for analysis (in the first instance) based on their sequences not overlapping other known non-defensin genes and having some sequence homology with other fish defensins. The sequences of these peptides, along with their alignment to the annotated zebrafish defensins are shown in Table 3.1.

Primers were designed to detect mRNA transcripts of the genes by PCR from 24hpf embryonic cDNA, however no expression was detected (data not shown). To avoid overlooking any adult-specific expression, the analysis was also repeated using cDNA harvested from whole adult fish, however transcripts were still not detected.

Due to time constraints, this work was not pursued any further. However, the 38 potential transcripts identified could present the opportunity for further work.

Peptide/predicted peptide	Sequence	Chromosome
Defbl1	MKPQSIFILL-VLVVLALHFKEN-EAASFPW-------SCASLSGVCRQGVCLPS-ELYF GPLGCG-KGFLCCVSHFL--	21
Defbl2	MKKLGMIIFITLLALFAGNVHNA-EVQIQNW-------TCG-YGGLCRR-FCFDQ-EYIV AHHGCP-RRYRCCAVRF---	21
Defbl3	MRTLGLIIFA-LLLLTASQAN---DTDVQRW-------TCG-YRGLCRK-HCYAR-EYMI GYRGCP-RRYRCCALRF---	15
1	-----MQIFACLLCFFCLIHLQN-QPARPGR-------SCTLSSGTCTVVHCFLYIQYSV ICKKHG-SQVLCCKVVFRSP	21
2	-----MKLFSSLSCFLSFLFQNDGHSFRLIWGQSVQWLFCKLLTGLCRWRFCRKL---FI AVGNCK-LRKTCCKTLKSQP	6
3	---MKLSYILLATCKESISLTSAPSPSESGYCPVLS--PCS-SQGLCYT-HCQKQ--THA NKYTCQ-TQRKCCKVQWHFL	14
4	-----MKISSSLLAACLLVCSRG-EPLSSIM--------CTLARGRCSSIRCCVT--WRK VVLFCGQNRKICCLKERYLE	21
5	-----MRPLIGAFSVICLSLRSE-----QKR--------CCSRIGQCRK-ICGYS-FHFR VIDLCNIVFKQCCRNATFQP	21

Table 3.1 Sequences of existing and predicted zebrafish defensins

Sequences were aligned using Clustal W2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Conserved cysteine residues are highlighted in red.

3.3.2 *defbl1* and *defbl2* have reported paralogues in an adjacent gene cluster

Since the publication of the Zou *et al.*, 2007 paper, paralogues to both *defbl1* and *defbl2* have been annotated in Ensembl (Zebrafish Zv8, Aug 2010). These genes are located adjacent to the zebrafish defensin cluster on chromosome 21 (Figure 3.1). To differentiate between these genes, I will refer to the 5' *defbl1/defbl2* cluster as "*defbl1.1* and *defbl2.1*" and the 3' cluster "*defbl1.2* and *defbl2.2*" (see Figure 3.1).

In order to gain insight into whether these genes are real paralogues or simply errors in the zebrafish genome assembly, their synteny was analysed. Supporting the notion that these are genuine sister genes (as opposed to an error in the assembly) is that the synteny surrounding the two gene clusters is different. Although both sets of genes are flanked by a cluster of sialidase genes, the number of genes annotated within these clusters varies (Figure 3.1), suggesting that divergence may have occurred following a duplication of this area. Figure 3.1 shows the presence of three sialidase (*neu*) genes 5' of *defbl2* at position 23.20Mb, compared to just one 5' to the second *defbl2* at position 23.40Mb. Paralogous genes can be expected to show some divergence (unless their founder duplication event occurred very recently). Although the transcribed sequences of *defbl2.1* and *defbl2.2* were identical, some evidence of divergence can be seen when comparing the sequences of *defbl1.1* and d*efbl1.2*. There are seven single nucleotide changes between the two gene transcripts (Figure 3.2a), however only two of these lie within the translated region of the gene. One of these two mutations is silent and the other leads to a point mutation in the signal sequence of the Defbl1 peptide (leaving the mature peptides identical)(Figure 3.2b). Since these changes were only single amino acids, it was not possible to differentially amplify the individual transcripts using RT-PCR. In addition, sequencing the gene yielded a single sequence identical to *defbl1.2*, with no duplicity being detected at the point mutation areas. This could indicate that *defbl1.1* is an error in the zebrafish assembly, but might also indicate differential expression of the genes.

The *defbl1.1*, *defbl2.1*, *defbl2.2* and *defbl3* genes have all been removed from the new zebrafish assembly (Zebrafish Zv9, November 2010), leaving just *defbl1.2* behind. Investigations are ongoing to determine the reasons behind this, however

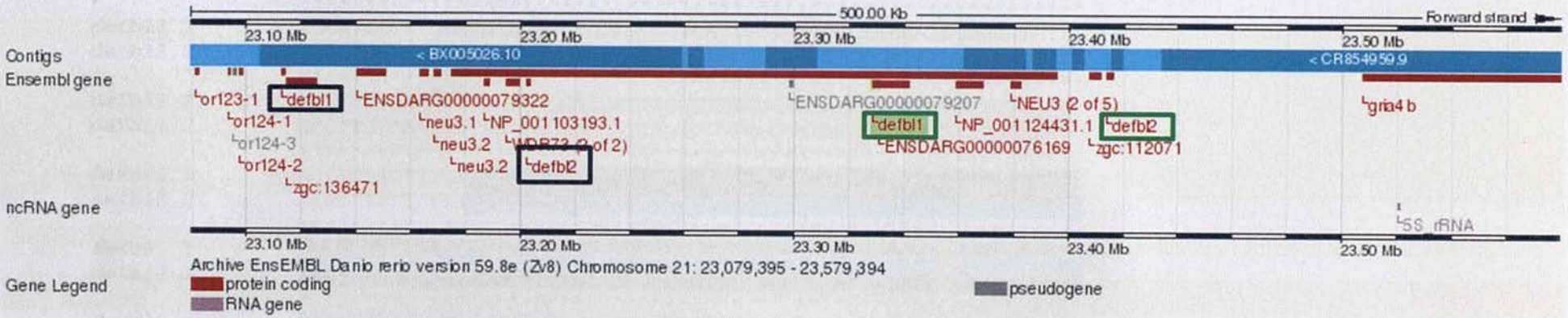
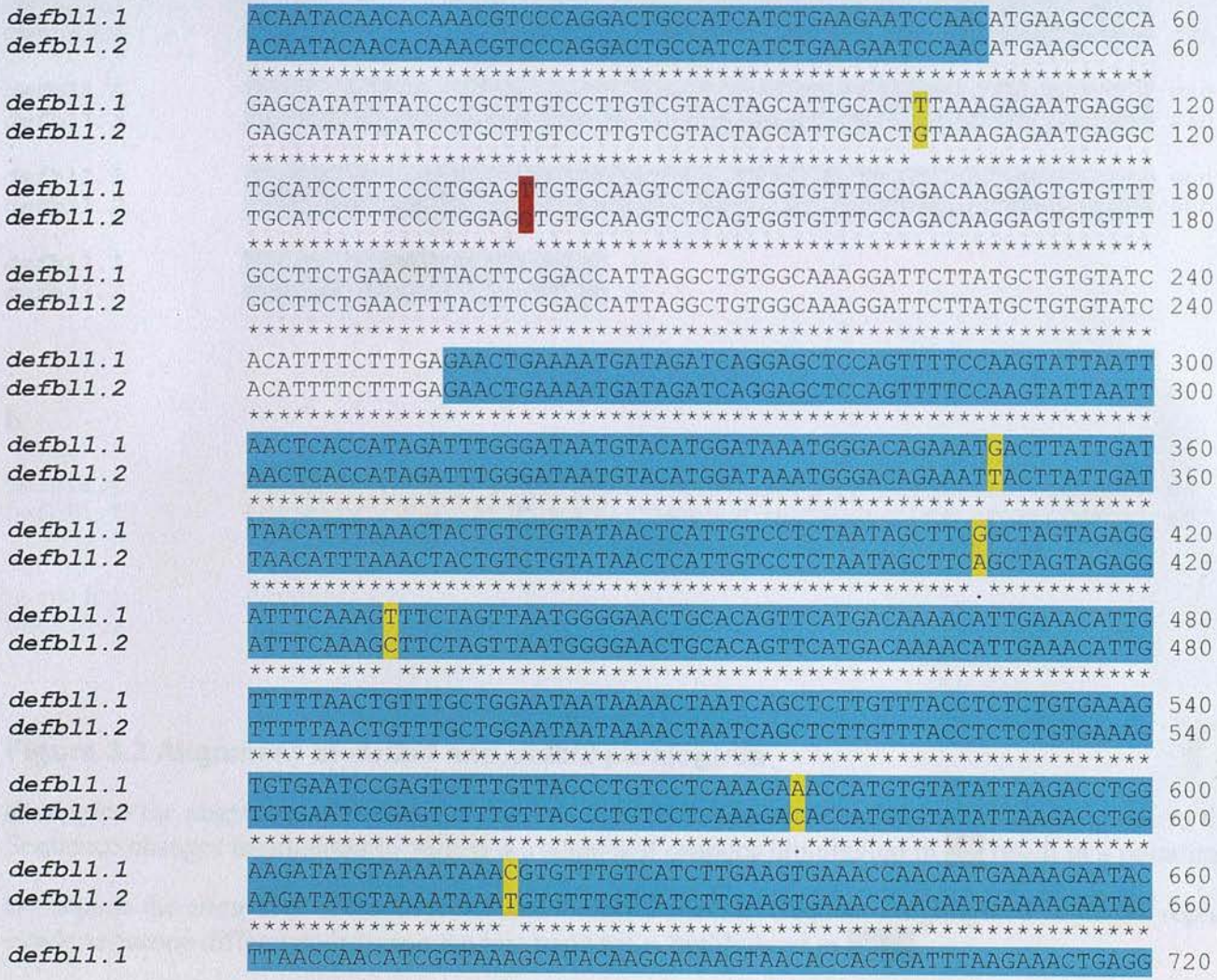


Figure 3.1 *defbl1* and *defbl2* and their paralogues

This figure depicts the 23.10-23.50Mb region of zebrafish chromosome 23 (according to Zebrafish Zv8, August 2010). Highlighted in **black** are *defbl1.1* and *defbl1.2* and highlighted in **green** are *defbl1.2* and *defbl2.2*. For clarity, the following unnamed genes are as follows (from 5' 23.10Mb to 3' 23.50Mb): zgc:136471: DNA polymerase Δ3; ENSDARG00000079322: chordin-like; NP_001103193.1: hypothetical sialidase, WDR73: WD repeat containing; ENSDARG00000076169: DNA polymerase Δ3; NP_001124431.1: chordin-like; zgc:112071: WD repeat containing region.

a


defbl1.1 ACAATACAACACAAACGTCCCAGGACTGCCATCATCTGAAGAATCCAACATGAAGCCCCA 60
defbl1.2 ACAATACAACACAAACGTCCCAGGACTGCCATCATCTGAAGAATCCAACATGAAGCCCCA 60
defbl1.1 GAGCATATTTATCCTGCTTGTCCTTGTCGTACTAGCATTGCACTTTAAAGAGAATGAGGC 120
defbl1.2 GAGCATATTTATCCTGCTTGTCCTTGTCGTACTAGCATTGCACTGTAAAGAGAATGAGGC 120
defbl1.1 TGCATCCTTTCCCTGGAGTTGTGCAAGTCTCAGTGGTGTTTGCAGACAAGGAGTGTGTTT 180
defbl1.2 TGCATCCTTTCCCTGGAGGTGTGCAAGTCTCAGTGGTGTTTGCAGACAAGGAGTGTGTTT 180
defbl1.1 GCCTTCTGAACTTTACTTCGGACCATTAGGCTGTGGCAAAGGATTCTTATGCTGTGTATC 240
defbl1.2 GCCTTCTGAACTTTACTTCGGACCATTAGGCTGTGGCAAAGGATTCTTATGCTGTGTATC 240
defbl1.1 ACATTTTCTTTGAGAACTGAAAATGATAGATCAGGAGCTCCAGTTTTCCAAGTATTAATT 300
defbl1.2 ACATTTTCTTTGAGAACTGAAAATGATAGATCAGGAGCTCCAGTTTTCCAAGTATTAATT 300
defbl1.1 AACTCACCATAGATTTGGGATAATGTACATGGATAAATGGGACAGAAATGACTTATTGAT 360
defbl1.2 AACTCACCATAGATTTGGGATAATGTACATGGATAAATGGGACAGAAATTACTTATTGAT 360
defbl1.1 TAACATTTAAACTACTGTCTGTATAACTCATTGTCCTCTAATAGCTTCGGCTAGTAGAGG 420
defbl1.2 TAACATTTAAACTACTGTCTGTATAACTCATTGTCCTCTAATAGCTTCAGCTAGTAGAGG 420
defbl1.1 ATTTCAAAGTTTCTAGTTAATGGGGAACTGCACAGTTCATGACAAAACATTGAAACATTG 480
defbl1.2 ATTTCAAAGCTTCTAGTTAATGGGGAACTGCACAGTTCATGACAAAACATTGAAACATTG 480
defbl1.1 TTTTTAACTGTTTGCTGGAATAATAAAACTAATCAGCTCTTGTTTACCTCTCTGTGAAAG 540
defbl1.2 TTTTTAACTGTTTGCTGGAATAATAAAACTAATCAGCTCTTGTTTACCTCTCTGTGAAAG 540
defbl1.1 TGTGAATCCGAGTCTTTGTTACCCTGTCCTCAAAGAAACCATGTGTATATTAAGACCTGG 600
defbl1.2 TGTGAATCCGAGTCTTTGTTACCCTGTCCTCAAAGACACCATGTGTATATTAAGACCTGG 600
defbl1.1 AAGATATGTAAAATAAACGTGTTTGTCATCTTGAAGTGAAACCAACAATGAAAAGAATAC 660
defbl1.2 AAGATATGTAAAATAAATGTGTTTGTCATCTTGAAGTGAAACCAACAATGAAAAGAATAC 660
defbl1.1 TTAACCAACATCGGTAAAGCATACAAGCACAAGTAACACCACTGATTTAAGAAACTGAGG 720

```
defbl1.2        TTAACCAACATCGGTAAAGCATACAAGCACAAGTAACACCACTGATTTAAGAAACTGAGG 720
                ************************************************************

defbl1.1        TAAAAAGTTGGGAAGAATACTGCATATATGTAACAGATTATGTTGAAATACAATTGTATT 780
defbl1.2        TAAAAAGTTGGGAAGAATACTGCATATATGTAACAGATTATGTTGAAATACAATTGTATT 780
                ************************************************************

defbl1.1        AAAAACAGGATATTCCTGTAGACATTTTAAATGTATGCATTCGGTGTACAAGGATCCTTG 840
defbl1.2        AAAAACAGGATATTCCTGTAGACATTTTAAATGTATGCATTCGGTGTACAAGGATCCTTG 840
                ************************************************************

defbl1.1        CAATTAAAATGTCTACTTCAGAATAACTTCAGCTAAAGAAAATGTCATAATATTGTTCTT 900
defbl1.2        CAATTAAAATGTCTACTTCAGAATAACTTCAGCTAAAGAAAATGTCATAATATTGTTCTT 900
                ************************************************************

defbl1.1        GTACTGTATCAAACTTTGAATGTC 924
defbl1.2        GTACTGTATCAAACTTTGAATGTC 924
                ************************
```

b

```
Defbl1.1        MKPQSIFILLVLVVLALHGKENEAASFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFL 60
Defbl1.2        MKPQSIFILLVLVVLALHGKENEAASFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFL 60
                ****************** ************************************

Defbl1.1        CCVSHFL 67
Defbl1.2        CCVSHFL 67
                *******
```

Figure 3.2 Alignment of *defbl1* and *defbl2* paralogues

(a) depicts the alignment of defbl1.1 and defbl1.2 mRNA. Sequence highlighted in blue represents the 3' and 5' untranslated regions. Sequence changes highlighted in yellow are silent and changes highlighted in red result in a point mutation.

(b) depicts the alignment of the Defbl1.1 and Defbl1.2 peptide. Sequence highlighted in yellow represents the signal sequence. The single sequence difference between the two peptides is highlighted in green.

since experimental data have shown that *defbl2* and *defbl3* are transcribed and both the Zv8 and Zv9 assemblies are categorised as "preliminary" , I will utilise the Zebrafish Zv8, August 2010 release of the genome in this thesis. Moreover, since I have not proven the existence of *defbl1.1* and *defbl2.1* and the mature peptide sequences are similar (*defbl1*) and identical (*defbl2*), the analysis in this thesis refers to a single *defbl1* and *defbl2*, based on the 3'cluster (at 23.40Mb).

3.4 EXPRESSION OF THE ZEBRAFISH DEFENSINS

Zou *et al.*, 2007 previously demonstrated the expression (at the RNA level) of *defbl1*, *defbl2* and *defbl3* in various tissues of the healthy zebrafish adult. In order to investigate whether expression levels were inducible in response to infection (as is seen with many mammalian defensins, see Section 1.4), it was decided to observe RNA levels in both healthy and infected zebrafish embryos.

3.4.1 Expression of zebrafish defensins in embryos

Zebrafish embryos presented an inviting model for this study, in particular because this would eliminate the need to perform invasive techniques on adult fish, reducing breeding stocks. In addition, since the defensins are part of the innate immune system, it is reasonable to expect them to be expressed in zebrafish embryos (which possess innate but not adaptive immunity at this stage).

RT-PCR was used to investigate the expression levels of the three genes in embryos aged 24hpf (hours post fertilisation) (Figure 3.3a). *defbl1* was the only gene detected in the early embryo, so to ascertain when *defbl2* and *defbl3* are first expressed, a panel of cDNA taken from larvae aged 3dpf, 5dpf, 6dpf and 7dpf and RT-PCR was performed again (Figure 3.3b). *defbl1* was present in all the tested stages, whereas *defbl2* and *defbl3* could only be detected in 6dpf and 7dpf embryos (faint bands for *defbl3* were verified by sequencing).

Since these experiments were performed on healthy embryos, it was reasoned that *defbl2* and *defbl3* might be inducible following embryonic/early larval infection.

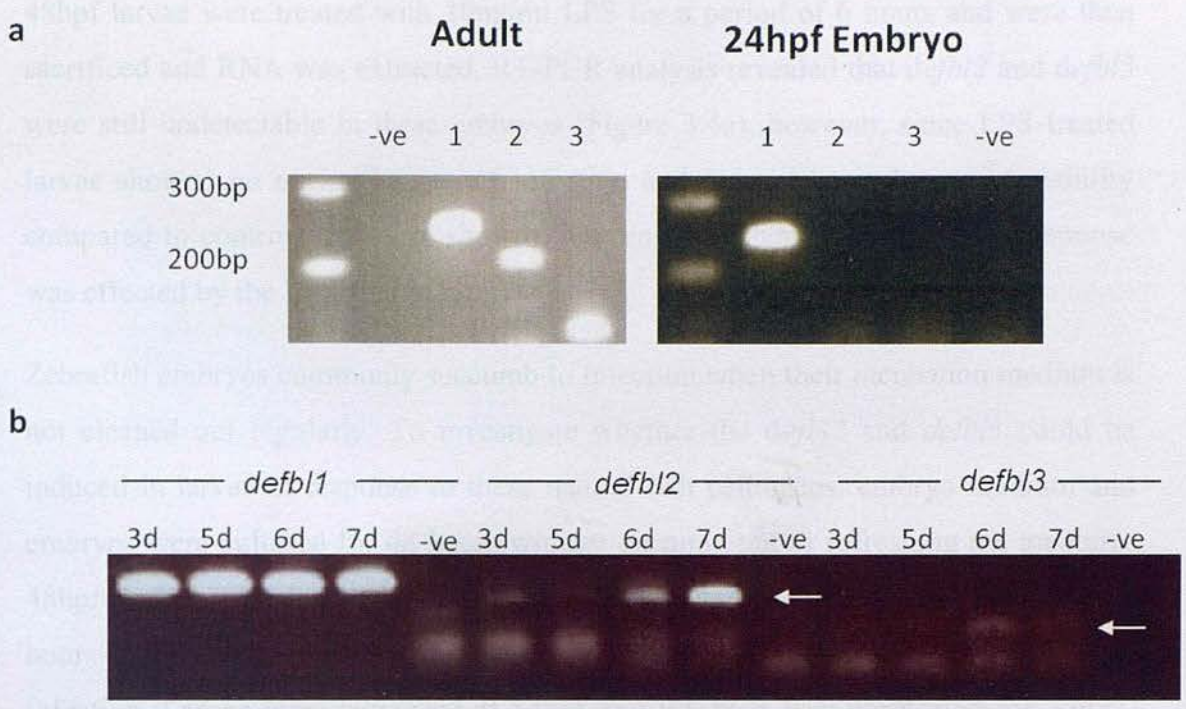


Figure 3.3 Embryonic expression of the zebrafish defensins

(a) shows the results of RT-PCR analysis looking for defbl1, defbl2 and defbl3 in cDNA from 24hpf embryos. A panel showing adult expression was included as a positive control. Bands were verified following gel purification by sequencing analysis (data not shown).
(b) depicts RT-PCR analysis carried out in larval embryos at 3dpf, 5dpf, 6dpf and 7dpf. The lower band in these gels represents primer dimer and the upper bands (white arrows) were verified by sequencing.

48hpf larvae were treated with 10ng/ml LPS for a period of 6 hours and were then sacrificed and RNA was extracted. RT-PCR analysis revealed that d*efbl2* and d*efbl3* were still undetectable in these embryos (Figure 3.4a), however, since LPS-treated larvae showed no outward signs of infection and did not have decreased viability compared to controls (data not shown), it is unclear whether any immune response was effected by the LPS-treatment.

Zebrafish embryos commonly succumb to infection when their incubation medium is not cleaned out regularly. To investigate whether the d*efbl2* and *defbl3* could be induced in larvae in response to these natural fish pathogens, embryo medium and embryos were cultured for 48 hours without cleaning out or refreshing the medium. 48hpf fresh larvae were then harvested, dechorionated and incubated for a further 6 hours in the cultured "dirty" medium, which showed clear fungal and bacterial infection. Larvae were harvested at 54hpf and RT-PCR was performed for *defbl1*, *defbl2* and *defbl3*. No expression for *defbl2* and *defbl3* was detected (Figure 3.4b). Survival of treated larvae was again, no different to the survival of the control treated larvae (data not shown), so it is unclear whether lack of infections was the reason that no induction was seen. This study suggests that a more robust method of infection should be optimised prior to repeating the investigation.

3.5 ANTIMICROBIAL ACTIVITY OF THE ZEBRAFISH DEFENSINS

The defining characteristic of the β-defensin family is the ability to kill or inhibit the growth of a broad spectrum of microbes. To address whether Defbl1, Defbl2 and Defbl3 are antimicrobial, synthetic preparations of the mature peptides (synthesised by collaborator Derek Macmillan, UCL) were tested *in vitro* against a panel of pathogens (Table 3.2). This panel was chosen to include a range of gram positive and negative bacteria and the fungus *Candida albicans*. The panel also included a number of opportunistic human pathogens (e.g. *Acinetobacter baumannii*) to dually investigate whether any of these peptides would have potential value as base peptides for the development of novel antibiotics. Finally, *Edwardsiella tarda* was also included as an established natural zebrafish pathogen to address the idea that the zebrafish defensins may have evolved to specifically target fish pathogens. It has

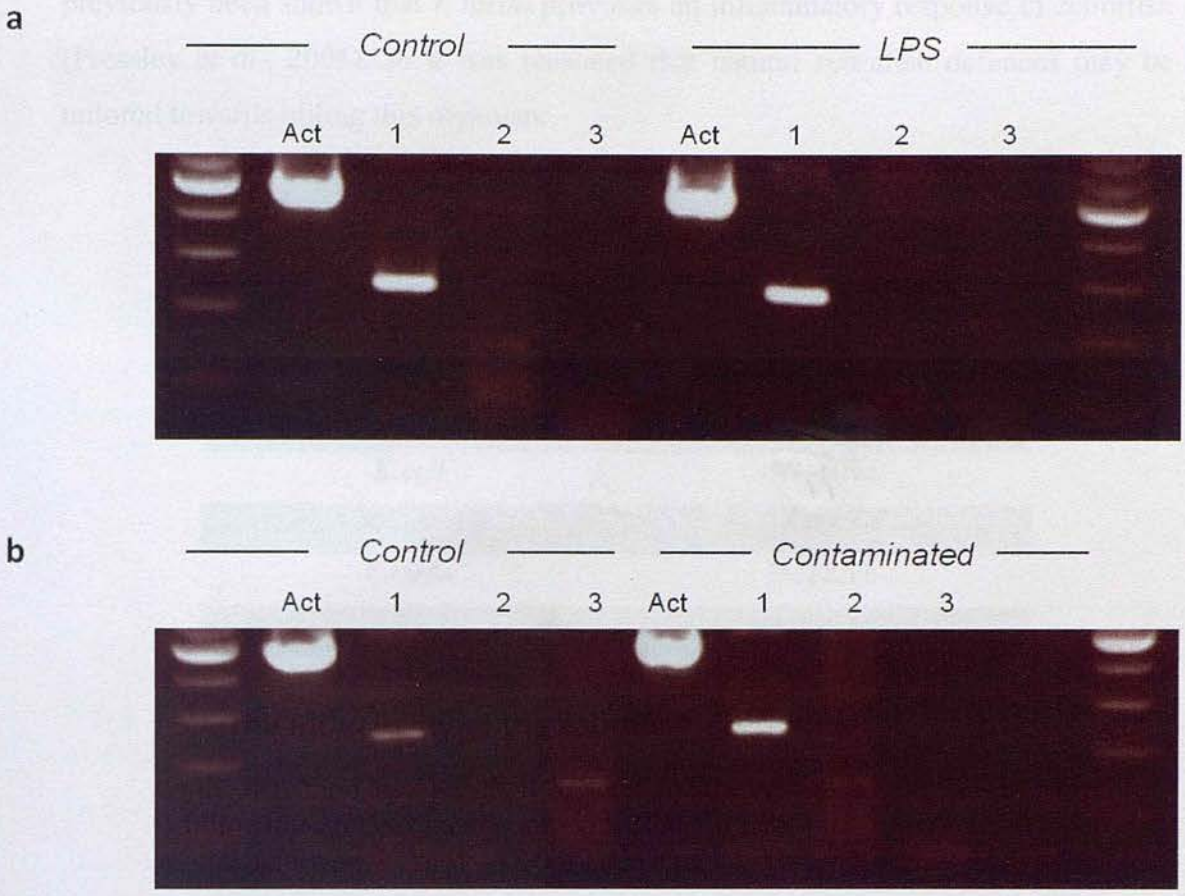


Figure 3.4 The inducibility of the zebrafish defensins

(a) shows the detection, by qRT-PCR of actin and the zebrafish defensins in 54hpf embryos following a 6 hour incubation with 10ng/ml LPS.
(b) shows the detection, by qRT-PCR of actin and the zebrafish defensins in 54hpf embryos following a 6hr incubation in bacteria and fungi-contaminated embryonic medium

previously been shown that *E.tarda* provokes an inflammatory response in zebrafish (Pressley *et al.*, 2005), so it was reasoned that natural zebrafish defences may be tailored towards killing this organism.

Organism	Gram status
P.aeruginosa	Negative
E.coli	Negative
A.baumannii	Negative
E.tarda	Negative
MRSA	Positive
E.faecalis	Positive
S.aureus	Positive
C.albicans	Fungus

Table 3.2 List of organisms used in zebrafish defensin antimicrobial assays

3.5.1 Antimicrobial activity of the zebrafish defensins against human and zebrafish pathogens

Mature peptide sequences, as reported by Zou *et al.*, 2007 (Table 3.3) were synthesised "in-house" (Defbl1, Defbl3; Derek Macmillan, UCL) or were purchased from United Peptide Corporation. Peptides were not folded following the reports (as summarised in Section 2.1) that antimicrobial activity is not dependent on 3D structure.

In vitro antimicrobial analysis was performed using Defbl1, Defbl2 and Defbl3 against a panel of microbes at 37°C (Figures 3.5-3.8). Minimum bactericidal concentration (MBC) values were determined to assess the minimum concentration of peptide required to kill 99.99% initial microbial inoculum.

Peptide	Sequence	ΔG	Charge
Defbl1	ASFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFLCCVSHFL	+4.01	+2
Defbl2	AEVQIQNWTC-GYGGLC-RRFCFDQEYIVAHHGCPRRYRCCAVRF	-6.76	+5
Defbl3	NDTDVQRWTC-GYRGLC-RKHCYAREYMIGYRGCPRRYRCCALRF	-5.12	+8

Table 3.3 Sequences of the mature zebrafish defensins
This table depicts the mature peptide sequences (following cleavage of the signal sequence) of the zebrafish defensins, as predicted in Zou *et al.*, 2007.

3.5.2 Antimicrobial activity of the zebrafish defensins against human and zebrafish pathogens

Defbl1 showed no activity against any of the strains tested at the highest tested concentration of 10μM (Figures 3.5-3.8). In contrast, Defbl2 showed weak antimicrobial activity (MBC = 10μM) only against *P.aeruginosa* (Figure 3.5), whereas Defbl3 showed notable activity against all tested strains, with MBCs ranging from 3.1 (±0.6) μM (*P.aeruginosa*, Figure 3.5) to 10 (±0.0) μM (*E.tarda*, Figure 3.7). No striking pattern in the specificity of activity of Defbl3 was observed and the peptide showed a moderate level of activity against both gram positive and negative bacteria, *E.tarda* and the fungus, *C.albicans*. A summary of the MBC values is shown in Table 3.4.

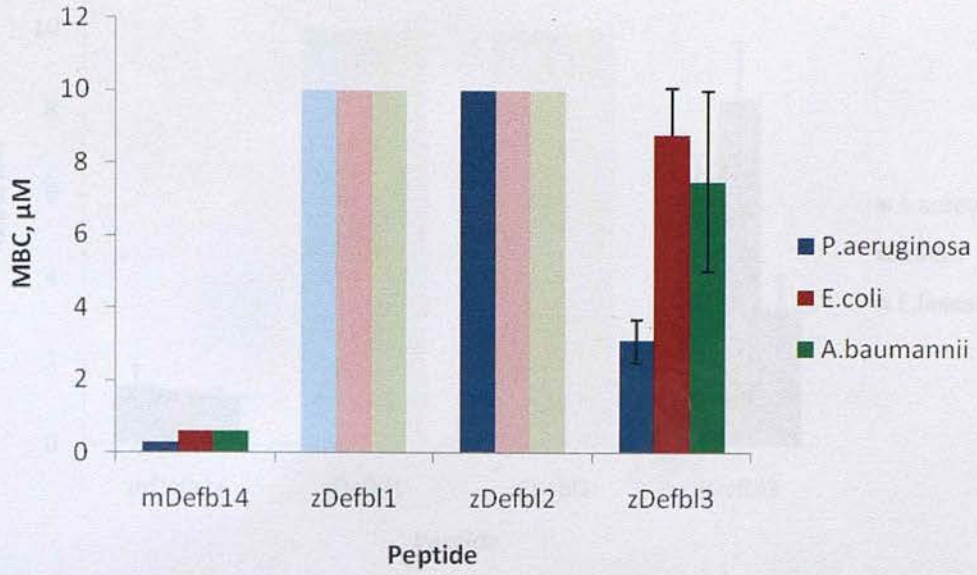


Figure 3.5 Bactericidal activity of the zebrafish defensins against gram negative bacteria

The graph depicts the antimicrobial activity (measured as MBC, µM) of the zebrafish defensins, as compared to the very potent murine Defb14 (described in Chapter 2). Data is based on three independent experimental repeats and error bars represent the standard error of mean (SEM). Where no error bar is depicted, SEM = 0.0.
Faded out bars denote peptides which did not achieve a MBC at the highest tested concentration.

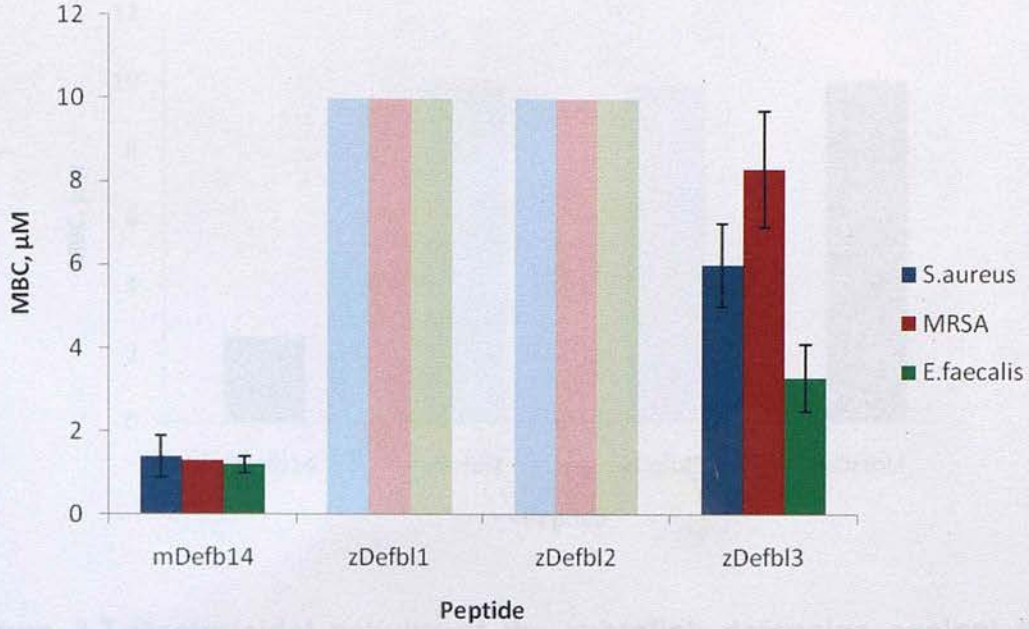


Figure 3.6 Bactericidal activity of the zebrafish defensins against gram positive bacteria

The graph depicts the antimicrobial activity (measured as MBC, µM) of the zebrafish defensins, as compared to the very potent murine Defb14 (described in Chapter 2). Data is based on three independent experimental repeats and error bars represent the standard error of mean (SEM). Where no error bar is depicted, SEM = 0.0.
Faded out bars (Defbl1 and Defbl2) denote peptides which did not achieve a MBC at the highest tested concentration.

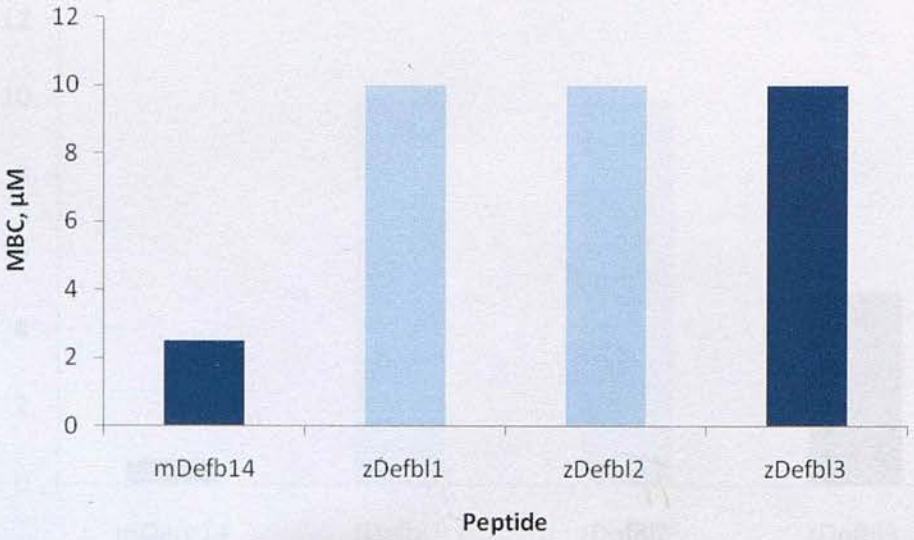


Figure 3.7 Bactericidal activity of the zebrafish defensins against the gram negative fish pathogen, *E.tarda*.

This graph depicts the bactericidal activity of the zebrafish defensins against a pathogen known to infect fish, *E.tarda*. Faded out bars indicate the peptide failed to achieve a MBC at the depicted concentration. Murine Defb14 has been shown to illustrate the MBC achieved by a very potent antimicrobial.
SEM for these peptides is 0.0

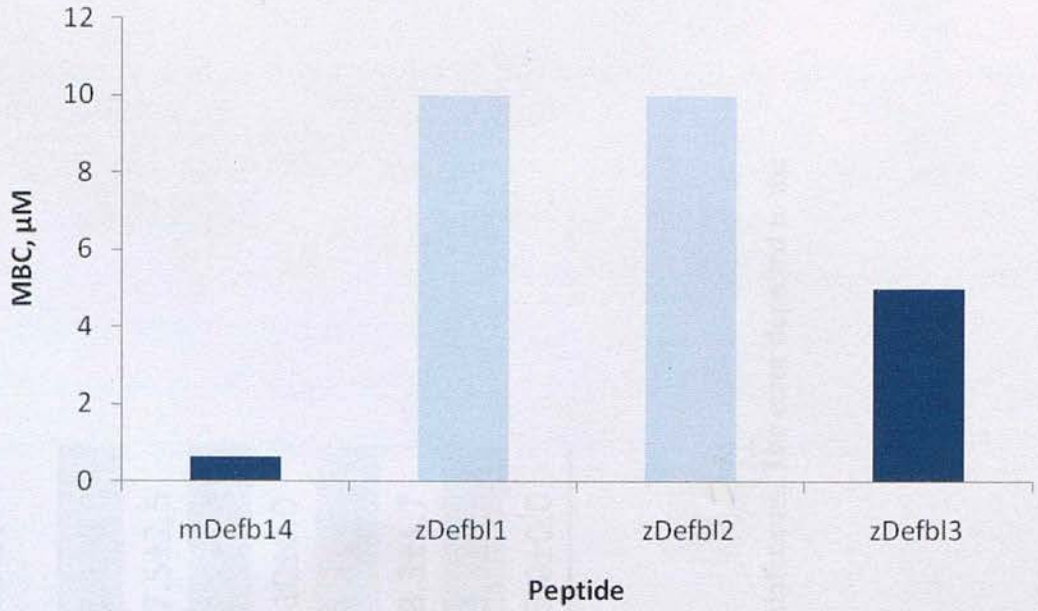


Figure 3.8 Antimicrobial activity of the zebrafish defensins against the fungus, *C.albicans*

This graph depicts the antimicrobial activity (measured using MBC,µM) of the zebrafish defensins against the fungus C.albicans. Faded out bars (Defbl1 and Defbl2) imply that the peptide did not achieve a MBC at the depicted concentration. Murine Defb14 has been included as an example of the MBC which can be expected when treating with a highly potent antimicrobial.
SEM for these peptides is 0.0.

	Organism	Defbl1 MBC, µM	Defbl2 MBC, µM	Defbl3 MBC, µM
Gm -ve	*P.aeruginosa*	>10	10±0.0	3.1±0.6
	A.baumannii	>10	>10	7.5±2.5
	E.coli	>10	>10	8.8±1.3
	E.tarda	>10	>10	10±0.0
Gm +ve	*S.aureus*	>10	>10	5.4±1.1
	MRSA	>10	>10	8.3±1.7
	E.faecalis	>10	>10	3.3±0.8
	C.albicans	>10	>10	5.0±0.0

Table 3.4 Summary of zebrafish defensin antimicrobial activity

The above table presents a summary of the antimicrobial activity detected for the zebrafish defensins. The error depicted is the SEM, calculated based on three independent experimental repeats.

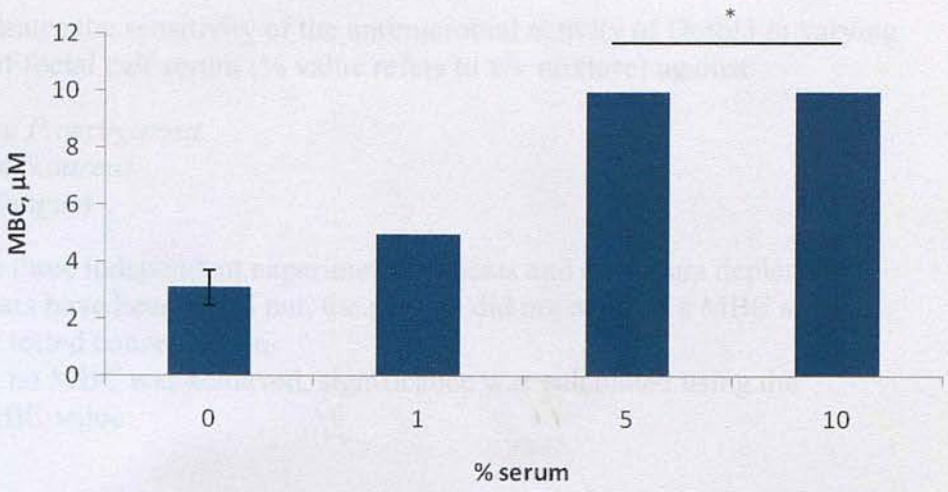
3.5.3 Serum and salt sensitivity of the antimicrobial activity of Defbl2 and Defbl3

To investigate the antimicrobial activity of Defbl2 and Defbl3 under more physiological conditions, *in vitro* antimicrobial assays were performed with the addition of serum and sodium chloride at varying concentrations. Defbl2 lost all activity (no MBC could be obtained) against *P.aeruginosa* when incubated in the lowest concentration of sodium chloride (50mM) and 1% (v/v) foetal calf serum (data not shown), indicating its activity is both salt and serum sensitive.

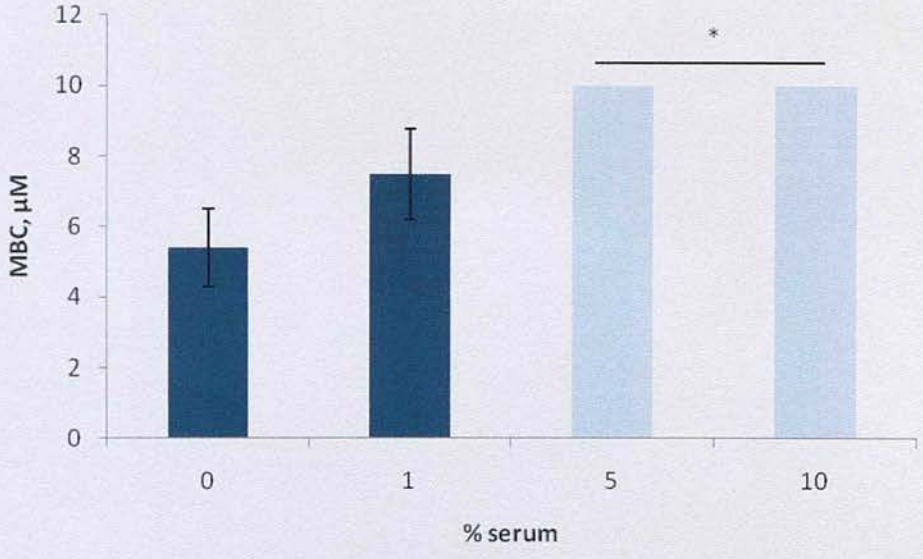
Defbl3 was tested for serum sensitivity against *P.aeruginosa*, as a representative of gram negative bacteria, *S.aureus* as a representative of gram positive bacteria and *C.albicans*. Although the addition of 5% and 10% serum significantly affected the MBC against all three strains (Figure 3.9a-c), lower level activity (MBC = 10 ± 0.0 μM) was still retained at 10% serum against both *P.aeruginosa* and *C.albicans* (Figure 3.9a,c), although activity was lost against gram positive *S.aureus* at serum concentrations of 5% upwards (Figure 3.9b).

The activity of Defbl3 alone was tested for salt sensitivity against *P.aeruginosa* as a representative gram negative organism and *S.aureus* as a representative gram positive organism. The bactericidal activity of Defbl3 showed a higher sensitivity to salt levels than serum, and its activity against *P.aeruginosa* was affected significantly upon incubation with 100mM NaCl (MBC = 10 ±0.0 μM) and activity was lost in 200mM NaCl (Figure 3.10a). Mirroring the results from the serum sensitivity experiments, Defbl3 activity against *S.aureus* showed an increased sensitivity, with significantly higher MBCs arising with the addition of just 50mM NaCl (MBC = 10μM ±0.0) and all activity was lost upon incubation with 100mM NaCl (Figure 3.10b).

(a)


MBC, µM
12
10
8
6
4
2
0
*
0
1
5
10
% serum

(b)


MBC, µM
12
10
8
6
4
2
0
*
0
1
5
10
% serum

(c)

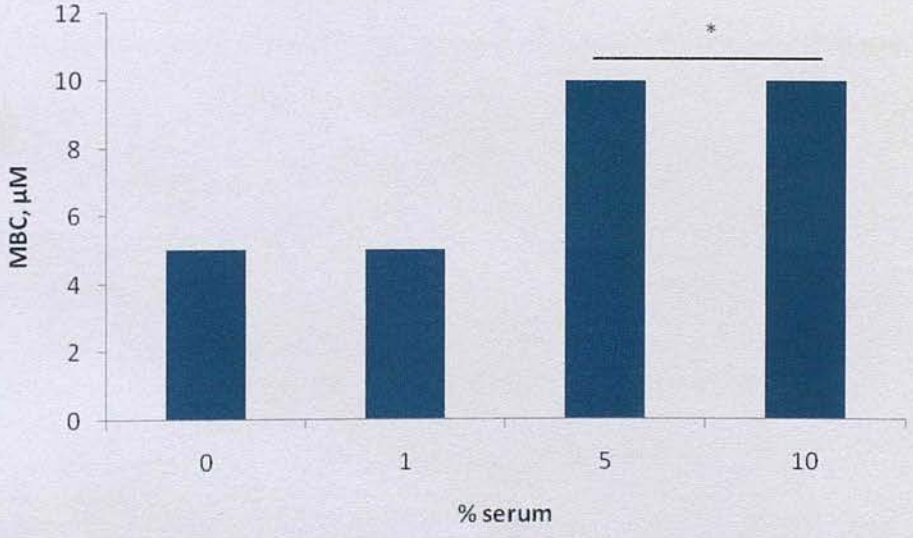

MBC, µM
12
10
8
6
4
2
0
*
0
1
5
10
% serum

Figure 3.9 Serum sensitivity of the antimicrobial activity of Defbl3 (figure on preceding page)

This figure illustrates the sensitivity of the antimicrobial activity of Defbl3 in varying concentrations of foetal calf serum (% value refers to v/v mixture) against:

(a) gram negative *P.aeruginosa*
(b) gram positive *S.aureus*
(c) *C.albicans* (fungus)

Data is based on three independent experimental repeats and error bars depict SEM values. Where bars have been faded out, the peptide did not achieve a MBC at the depicted highest tested concentration.
* $p<0.05$, where no MBC was achieved, significance was calculated using the highest tested MBC value.

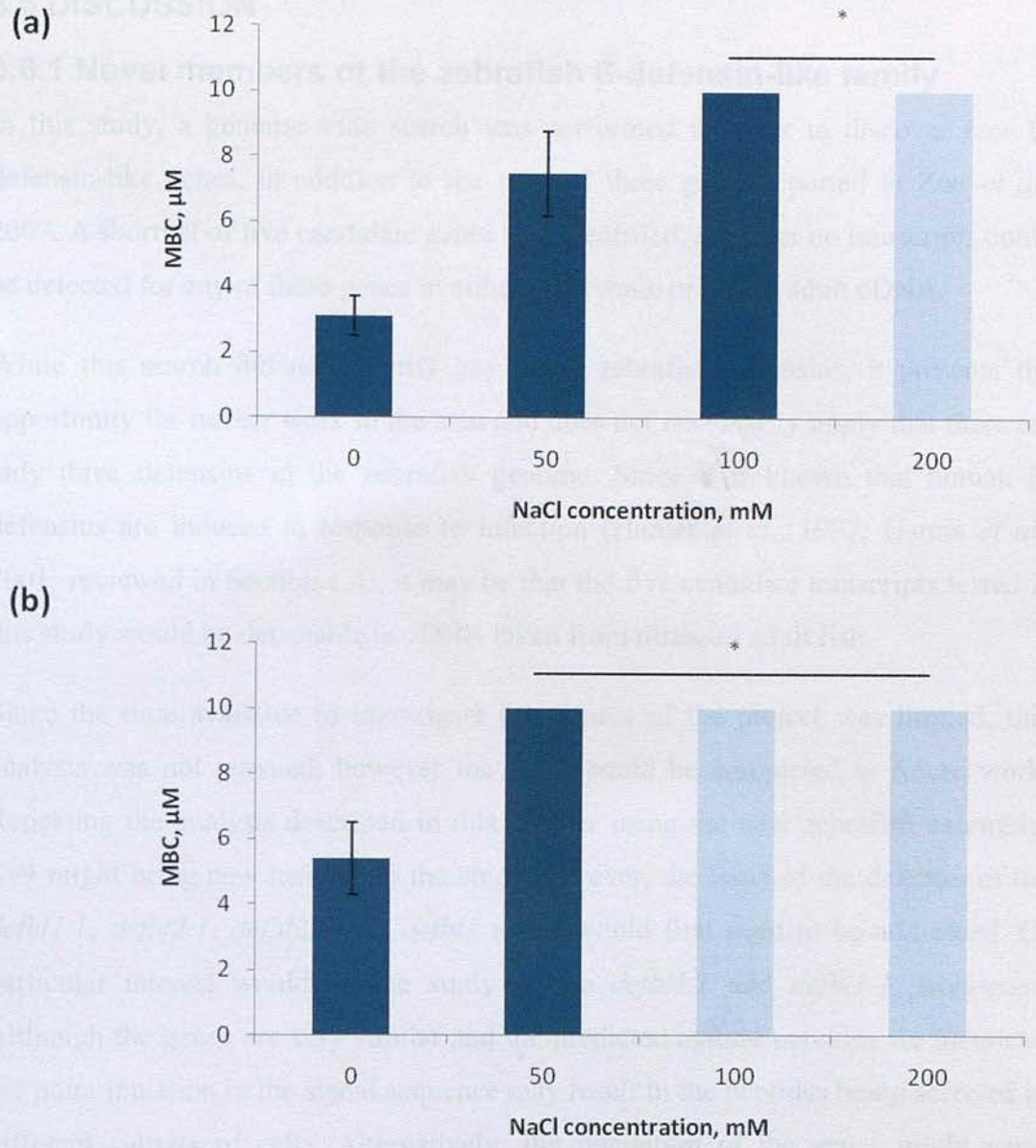


Figure 3.10 The salt (NaCl) sensitivity of the antimicrobial activity of Defbl3

This figure illustrates the sensitivity of the antimicrobial activity of Defbl3 in varying concentrations of NaCl salt against:

(a) gram negative *P.aeruginosa*
(b) gram positive *S.aureus*

Data is based on three independent experimental repeats and error bars depict SEM values. Where bars have been faded out, the peptide did not achieve a MBC at the depicted highest tested concentration.
* $p<0.05$, where no MBC was achieved, significance was calculated using the highest tested MBC value.

3.6 DISCUSSION

3.6.1 Novel members of the zebrafish β-defensin-like family

In this study, a genome-wide search was performed in order to discover new β-defensin-like genes, in addition to the original three genes reported in Zou *et al.*, 2007. A shortlist of five candidate genes was identified, however no transcripts could be detected for any of these genes in either embryonic or whole adult cDNA.

While this search did not identify any novel zebrafish defensins, it presents the opportunity for further work in the area and does not necessarily imply that there are only three defensins in the zebrafish genome. Since it is known that human β-defensins are induced in response to infection (Harder *et al.*, 1997; Garcia *et al.*, 2001; reviewed in Section 1.4), it may be that the five candidate transcripts tested in this study would be detectable in cDNA taken from diseased adult fish.

Since the time available to investigate this aspect of the project was limited, this analysis was not pursued; however the study could be completed in future work. Repeating the analysis described in this chapter using the new zebrafish assembly, Zv9 might bring new insights to the study however, the issue of the deletion of the *defbl1.1*, *defbl2.1*, *defbl2.2* and *defbl3* genes would first need to be addressed. Of particular interest would be the study of the *defbl1.1* and *defbl1.2* paralogues. Although the genes are very similar and the predicted mature peptides are identical, the point mutation in the signal sequence may result in the peptides being secreted in different subsets of cells. Alternatively, the regulation of the genes might vary, providing alternative roles for the genes. A further explanation could be that zebrafish show copy number variation (CNV) at this locus. Copy number variation has been reported at the human 8p23.1 locus (Hollox *et al.*, 2003) and this type of variation is notoriously difficult to sequence accurately. Since the zebrafish genome sequence is not based on the sequence of a single homogenous zebrafish, it is possible that the confusion arising in this area is down to copy number variation at this locus between the different fish sampled.

3.6.2 Expression of the zebrafish defensins

Prior to this study, Zou *et al.*, 2007 found that *defbl1*, *defbl2* and *defbl3* are expressed in a variety of healthy adult tissues. Oehlers *et al.*, 2011 extended this study and detected the expression of all *defbl1* and *defbl3* in zebrafish larvae. In the work described in this chapter, I have shown that *defbl1* can be detected in the early 24hpf embryo and that *defbl2* and *defbl3* can be detected in 6-7dpf larvae.

I reasoned that although *defbl2* and *defbl3* were not detectable in the early embryo, they might still be inducible in the 24-48hpf embryo following exposure to microbial stimuli. To investigate this, embryos were first treated with a 10ng/ml LPS solution and expression was examined. Watzke *et al.*, 2007 demonstrated that immersion of zebrafish larvae in LPS could induce the key genes in the innate immune response including IL-1β and TNF-α, so this experimental technique was used. Again, *defbl2* and *defbl3* were not detected in cDNA from these larvae, however analysis of expression of IL-1β and TNF-α (using the primers from Watzke *et al.*, 2007) revealed expression of these genes in both control and treated larvae (data not shown). Bleaching and dechorionating control early embryos and growing in sterile, autoclaved medium did not remove the presence of these bands (data not shown). Since it was not possible to prove that the LPS induced any response in the treated larvae, it is not possible to assert that *defbl2* and *defbl3* are uninducible at this stage. Since the Watzke *et al.*, 2007 study was carried out in younger (24hpf) embryos, it is possible that a higher concentration of LPS would be required for this experiment to be informative.

The induction of *defbl2* and *defbl3* was also investigated following treatment of 48hpf larvae with a mixture of bacteria and fungi cultured from contaminated embryo medium. As seen with the LPS study, no induction of *defbl2* and *defbl3* was observed. This experimental technique was chosen so that "natural" (within the confines of the fish facility) fish pathogens could be tested. I hypothesised that these microbes would be more likely to induce an immune response than any cultures of lab strain bacteria, however no induction was observed.

While these two studies indicate that *defbl2* and *defbl3* are not inducible in young larvae, it is possible that they are inducible in response to a specific, as yet untested

stimulus. For example, Proud *et al.*, 2004 demonstrated that *HBD2* is inducible in epithelial cells *in vivo* in response to rhinovirus infection. It is possible that *defbl2* and *defbl3* could be virally induced, in which case this study would not have identified the induction. The work described in this thesis should therefore be viewed as preliminary findings which could be expanded in future studies.

A major weakness of this work was the inability to confirm infection using infection markers. Analysis of the EST Profile of both IL-1β and TNF-α using Unigene suggests the genes are normally expressed in the zebrafish embryo and larva. To resolve the problem of infection confirmation, a number of steps could be taken. Although IL-1β and TNFα may normally be expressed at low levels in developing larva, Watzke *et al.*, 2007 have shown the genes to be inducible. It is therefore probable that any changes in gene expression could be examined quantitatively using qRT-PCR. Analysing expression of treated embryos in comparison to control embryos would result in a fold change which could be used to identify any induction of the immune response.

Alternatively, Stockhammer *et al.*, 2009 published a transcriptome modelling study, analysing changes in expression of innate immunity genes in response to *Salmonella* species infection. A set of genes could be chosen from this analysis to create a panel of genes known to be induced following bacterial infection.

Finally, the infection of larvae GFP-tagged bacteria would allow for time course experiments to be carried out. This would allow the infection to be visualised within the larval tissues before RNA extraction and analysis.

An alternative approach to examining the role of *defbl1*, *defbl2* and *defbl3* in zebrafish embryos and young larvae would be to employ morpholino technology. This would allow for the observation of the effects of a transient knockdown of the genes on fish survival in the presence of a variety of organisms. This could give insight into the *in vivo* immune function of these genes.

3.6.3 Antimicrobial activity of the zebrafish defensins

This analysis used a series of *in vitro* assays testing synthetic preparations of the zebrafish defensins against a panel of microbes. The work indicated that Defbl1 is inactive against all tested strains and that Defbl2 shows a low level activity specific to *P.aeruginosa*, whereas Defbl3 shows moderate antimicrobial activity against the full range of tested microbes.

The inactivity of Defbl1 may be explained in a number of ways. It is possible that the peptide is not antimicrobial and that instead, it has takes on another role in zebrafish immunity, for example, a role in the chemoattraction of immune cells to sites of infection, as is the case with Defb14 (Taylor *et al.*, 2008). Since *defbl1* mRNA was previously detected in a number of zebrafish tissues frequently exposed to bacteria, for example the gut and gill (Zou *et al.*, 2007), this presents a viable option for the peptide's function. Alternatively, the peptide may have evolved specific activity to one type of microbe, untested and therefore undetected in this assay. In particular, it should be noted that the organisms tested in this study were grown and assayed at 37°C. It may be more informative in future studies to test fish-specific organisms at the physiological zebrafish temperature of 28°C.

However, it is equally possible that Defbl1 is antimicrobial, but that the activity has not been detected in this *in vitro* experiment. A recent paper on the human β-defensin HBD1, showed that its low level activity can be drastically improved against a range of commensal bacteria and fungi under reducing conditions (Schroeder *et al.*, 2011). This work illustrates the importance of the chemical conditions required for antimicrobial activity. It is possible, therefore, that Defbl1 could display antimicrobial activity *in vivo* and that its inactivity in my assays is a product of testing *in vitro*. However, since many vertebrate peptides are antimicrobially active under *in vitro* conditions, it is impossible to predict whether Defbl1 is truly inactive or not without further experimentation (potentially *in vivo*).

In addition, the inactivity of Defbl1 might be explained by the fact that the peptide tested in this assay is synthetic. The test peptide was not folded post-production, and in addition is lacking any posttranslational modifications which might occur *in vivo*. Since the full requirements and mechanisms of antimicrobial activity are unknown,

this may have contributed to the inactivity of Defbl1. However, given the combined low charge and hydrophobicityof Defbl1, it is also arguable that the peptide is antimicrobially inactive *in vivo*.

Defbl2 showed singular weak activity against *P.aeruginosa*. While the same issues encountered with Defbl1 of synthetic structure and *in vitro* testing also apply to this peptide, it may also highlight another problem with the concept of synthesising peptides chemically. All three defensin peptides analysed in this study were synthesised as mature peptides, based on the signal sequence predictions made by the software Signal P 3.0. While the sequences for Defbl1 and Defbl3 showed clearcut signal sequence cleavage sites, Defbl2 contained two potential cleavage sites (Figure A1.1, A1.2). For the purposes of this study, the shorter peptide was chosen due to its similarity to the sequence of Defbl3 and previous publication in Zou *et al.*, 2007. However, since this decision was based on homology and probability, it is entirely possible that the synthetic Defbl2 peptide tested in this assay was shorter than the physiological mature peptide. In Chapter 2, I illustrated the importance short amino acid sequences can have on bactericidal activity within the context of a larger molecule, so it may be that the addition of the missing N-terminal amino acids of the Defbl2 mature peptide would improve its *in vitro* antimicrobial activity.

In order to definitively determine the correct sequence of Defbl2, it would be necessary to purify the peptide from adult zebrafish. Work aimed at isolating antimicrobial peptides from whole zebrafish extracts is currently ongoing (D. Campopiano, personal communication), and this work may uncover the true structure of Defbl2.

Supporting the notion that Defbl2 has more potent antimicrobial activity *in vivo* is the observation that the gene could only be detected in the gut of healthy adult zebrafish (Zou *et al.*, 2007). Since the gut is heavily laden with antimicrobial commensals, it is possible that Defbl2 is expressed in this region in response to and in order to control the microbial load.

Defbl3 showed moderate activity *in vitro* when compared to other potent mammalian antimicrobials such as HBD3. However, it is again possible that the *in vivo* activity

of this peptide might be improved over the results observed in this analysis. One major reason for this is the discrepancy between the temperature at which the antimicrobial assays were carried out (37°C) and the temperature at which Defbl3 would function *in vivo* (28°C). It might be that the peptide's activity becomes more (or less) potent at this lower temperature. There has been some evidence to suggest that bacteria alter the chemical composition of their cell membranes in response to different temperatures, with *P.aeruginosa* being reported to alter its membrane hydrophobicity following a reduction to 28°C (Hori *et al.*, 2009). Since the β-defensins' mode of action appears to be dependent on a delicate balance of charge and hydrophobic interactions with the membrane, then it is reasonable to suppose that altered cell wall composition could alter antimicrobial activity. In order to gain better insight into this, the antimicrobial assays could be repeated *in vitro* at 28°C and any differences in activity could be observed. In addition, this repeated assay could provide interesting insights to the issues of serum and salt sensitivity. For example, the serum sensitivity observed may have occurred due to direct inhibition of the peptide, or alternatively degradation. Peptide degradation might be less efficient at a lower temperature, resulting in a lower serum sensitivity. The issue of peptide degradation could be addressed in further work by analysis of the peptides in a, post-antimicrobial assay using mass spectrometry.

In summary, this analysis revealed that Defbl3 has broad spectrum antimicrobial activity, suggesting that the zebrafish defensin family may have an important role in zebrafish immunity. Although the peptide is not as potent an antimicrobial as HBD3, it still possesses solid, broad spectrum antimicrobial activity. The peptide may, therefore, provide insights for future work in the field of antibiotic development. In particular, its consistent activity against a range of different types of microbes suggests that it might be useful to studies aiming to produce novel broad-spectrum antibiotics.

3.7 CONCLUSIONS AND FUTURE DIRECTIONS

In this chapter, the expression and antimicrobial role of the zebrafish defensins was investigated. While this work did not result in the identification of any novel zebrafish defensin family members, it was shown that *defbl1* is expressed in early zebrafish embryos, whereas *defbl2* and *defbl3* are only expressed in older larvae. Furthermore, this work suggested that *defbl2* and *defbl3* may not be inducible in response to the stimuli tested in larval fish.

In addition, a detailed analysis of the *in vitro* antimicrobial activity of synthetic zebrafish defensin preparations was performed, suggesting that Defbl3 has potent antimicrobial activity against a range of different bugs.

The findings in this chapter are of value in two different areas. Firstly, the findings support the mounting evidence that the defensins are important in the immune system of zebrafish, and by association, other teleosts. In addition, the work described has identified Defbl3 as a broad spectrum antimicrobial peptide which may have relevance to future studies attempting to develop non-human defensins for use as clinical antimicrobial drugs.

Both of these areas of interest present interesting avenues for future research. In order to further understand the role of the defensins in zebrafish immunity, studying the action of the peptides *in vivo* might prove to be beneficial. Producing knock out transgenics for the three genes would allow for detailed infection studies to take place, where increased illness, or increased recovery times might indicate the importance of the genes in the pathology of disease. However, since it is unknown whether there are additional members of the defensin family in the zebrafish genome, this study might become ambiguous if unknown redundant members of the family are able to compensate for the lack of antimicrobial activity a knockout might produce.

To study further the question of whether the zebrafish defensins are inducible, detailed studies could be carried out in adult fish. A fully controlled experiment could involve tank mates being separated and groups being subjected to intraperitoneal injections of varied preparations of different classes of known fish

pathogens and commensals. It could then be ascertained whether the infections produced higher levels of defensin expression with respect to healthy, untreated tankmates. Alternatively, some success has been observed in infection studies where single scales are removed from the skin of adult fish, allowing fish to be infected via the wound upon immersion in a solution containing various microbes (Pressley *et al.*, 2005). This would present a less invasive method to approach the experiment.

In conclusion, the work in this chapter presents an interesting introduction to the zebrafish defensins, investigating both their suitability as candidates for novel therapeutics as well as beginning to define their role in the innate immunity of zebrafish. Ultimately, gaining a better understanding of zebrafish immunity might have implications as far reaching as the fishing industry, where an improved knowledge of fish immunity might lead to genetic engineering to produce commercial fish more resistant to disease.

Chapter 4: The Role of *defbl1* in Development in *Danio rerio*

"One sometimes finds what one is not looking for"

Sir Alexander Fleming

4.1 PREFACE

In Chapter 3, the antimicrobial properties of the β-defensin-like peptides were investigated. The study indicated that *defbl1* is the only member of the defensin family expressed in zebrafish embryos, yet a synthetic preparation failed to show any antimicrobial activity *in vitro*. In this chapter, I investigate the role of *defbl1* in greater depth, in order to establish its role in the early zebrafish embryo, with particular emphasis on establishing whether the peptide has a potential role in early zebrafish development.

4.1.1 Zebrafish development: an overview of the first 24 hours

Zebrafish embryonic development occurs rapidly, in well-documented stages as described in Kimmel *et al.*, 1995. An overview of this comprehensive study is described below.

Eggs are fertilised *ex vivo* and begin as a single cell attached to a large yolk mass (Figure 4.1a). This initially undergoes rapid, synchronous cell divisions, with divisions occurring roughly every 15 minutes (Kimmel *et al.*, 1995). From approximately 2.25hpf onwards (Figure 4.1c), the group of cells (the "blastodisc") begin to lose their cell cycle synchrony. This marks entry into the midblastula transition (MBT) where the cells become motile and embryonic transcription initiates (Kimmel *et al.*, 1995). Epiboly (Figure 4.1d), one of the first cellular rearrangements, begins in the late blastula stage, where the cells of the blastodisc thin and radiate over the surface of the embryonic yolk, until the whole surface is covered.

When the embryo reaches 50% epiboly, it enters the gastrula period of development (Figure 4.1e), where the first large-scale morphogenic events occur. During gastrulation, the undifferentiated blastula cells undergo a series of movements and rearrangements in order to form three embryonic germ layers (the endoderm, ectoderm and mesoderm) which ultimately give rise to a fully patterned embryo (reviewed in Rohde and Heisenberg, 2007). The cell movements driving vertebrate gastrulation are evolutionarily conserved (Solnica-Krezel, 2005) and result in the establishment of the dorsal-ventral and anterior-posterior axes.

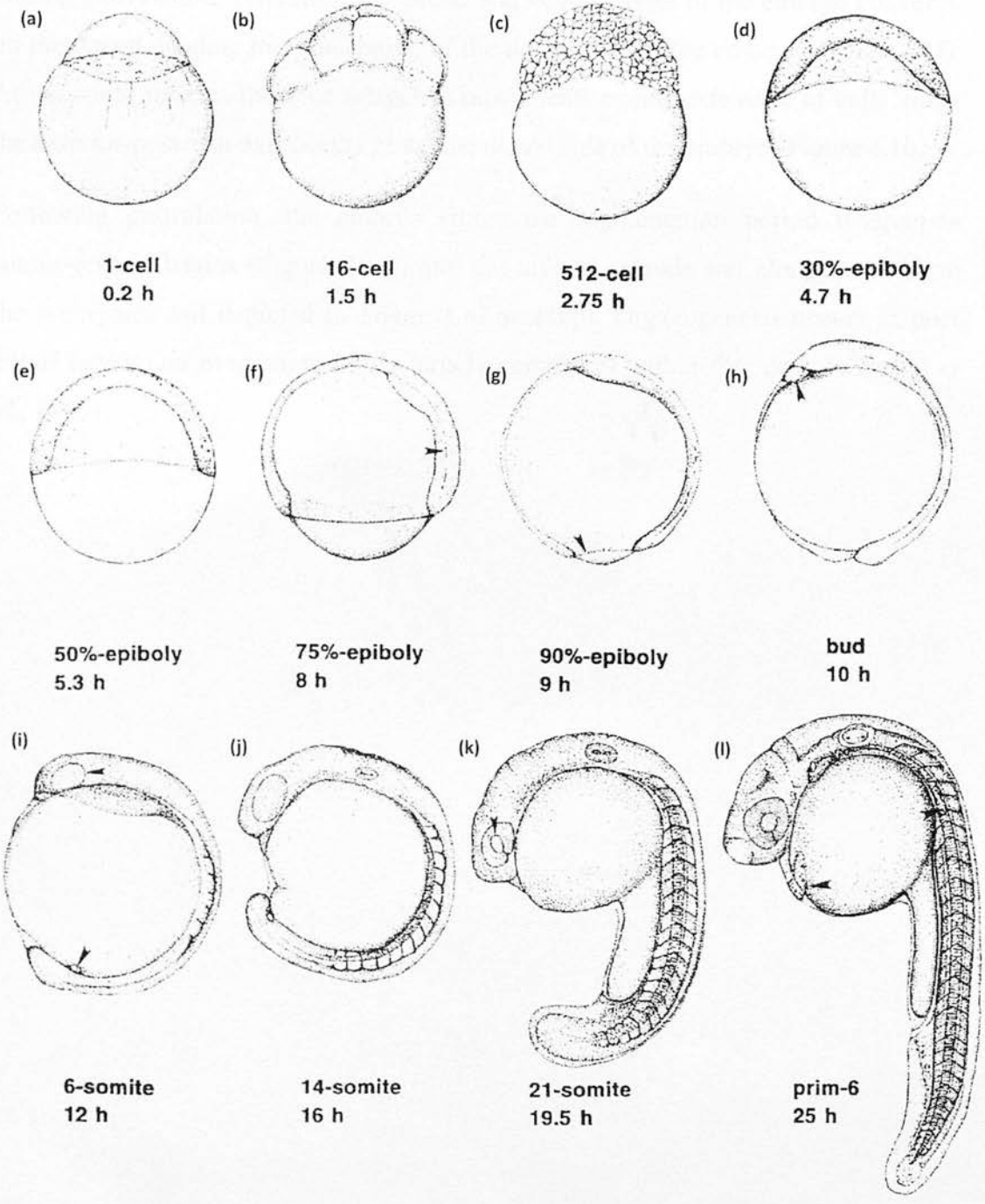


Figure 4.1 Zebrafish developmental stages (edited from Kimmel *et al.*, 1995)

This figure depicts "camera lucida" sketches of the developing embryo at various stages. Embryos are orientated are shown from the lateral side, with the dorsal side on the right. Embryonic stages are labelled according to their anatomical stage and the timepoint at which this developmental stage is reached.

During gastrulation, cells from the lateral and ventral edges of the embryo converge on the dorsal, leading to a thickening of the dorsal side of the embryo (Figure 4.1f). At the same time as these convergence movements occur, extension of cells along the anterior-posterior axis occurs along the dorsal side of the embryo (Figure 4.1h).

Following gastrulation, the embryo enters the segmentation period whereupon somitogenesis begins (Figure 4.1i,j) and the tailbud extends and elongates to form the embryonic tail depicted in Figure 1.4l at 24hpf. Organogenesis occurs in post 24hpf larvae and morphogenesis is largely completed within five days (Kimmel *et al.*, 1995).

4.2 AIMS

The main aim of this chapter was to investigate the role of *defbl1* in zebrafish embryos in the light of the findings of Chapter 3, where I could not detect an antimicrobial function for the Defbl1 peptide, despite detecting expression in the early embryo. In order to gain insight into the role of the gene, it was decided to investigate:

1. the effects of transiently knocking down *defbl1* expression using morpholino oligonucleotides
2. wholemount in situ hybridisation (WISH)
3. genome-wide expression analysis in *defbl* knockdown mutants

4.3 *DEFBL1* EXPRESSION

4.3.1 *defbl1* is expressed in the early embryo

As described in Chapter 3, analysis using RT-PCR revealed *defbl1* is the only documented zebrafish defensin to be expressed in the early embryo at 24hpf. To investigate the expression pattern in further detail, RT-PCR was performed on embryos harvested between 8hpf and 18hpf. Transcripts for *defbl1* were detectable from 12hpf (in this case, 12hpf embryos were staged at tail bud stage), and expression was detected continuously after this time point (Figure 4.2). In order to determine the location of the transcripts, in situ hybridisation was performed on fixed 13hpf and 24hpf embryos. Expression of *defbl1* was difficult to observe at 13hpf and no difference was observed between embryos stained with a *defbl1* probe and the corresponding sense probe (data not shown), indicating that expression levels are too low to detect. However, at 24hpf, *defbl1* appeared to localise to the embryonic head. From a lateral view, the staining is situated just outside (dorsal) of the developing eye (Figure 4.3b,c), appearing to localise to the diencephalon. When viewed from directly above the embryonic head, it can be seen that the staining localises to two symmetrical sites seemingly at the border of the telencephalon and the eye (Figure 4.3a,d).

4.3.2 Transient knockdown of *defbl1* causes multiple developmental defects

To gain insight into the function of *defbl1* in the early embryo, transient knockdowns were performed using morpholino technology. Morpholino oligonucleotides are short, single-stranded nucleotides possessing a modified DNA backbone comprising a six-carbon "morpholine" ring to replace the pentose ring observed in the nucleic acids. In addition, the oligomerising phosphate bonds seen in DNA are substituted with non-ionic, lower-charged phosphodiamidate links in the morpholino (Figure 4.4a) (Summerton, 2007). Morpholinos are widely used to transiently knock down gene expression in zebrafish and other organisms by binding mRNA transcripts (by Watson-Crick base pairing) to physically (sterically) block either translation or correct splicing, in a sequence-dependent manner. The modifications to the morpholino backbone enhance the stability of the oligomers *in vivo* and minimise

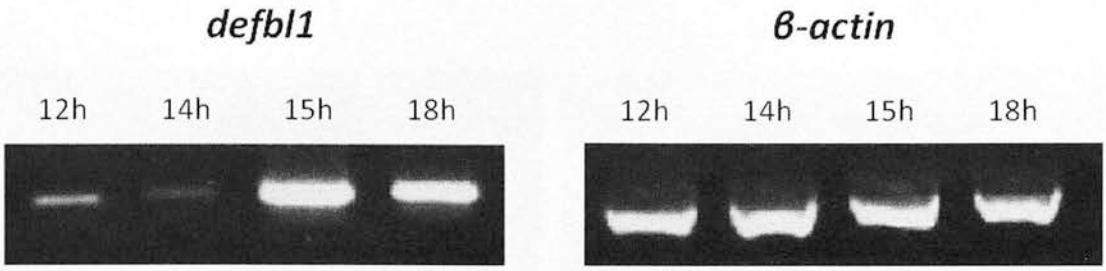


Figure 4.2 Expression of *defbl1* at different developmental stages

Embryos harvested at 12,14,15 and 18hpf were analysed for *defbl1* and *β-actin* expression (positive control). Embryos were staged prior to sacrificing and 12hpf corresponded to the tailbud stage of development (as described in Kimmel *et al.*, 1995)

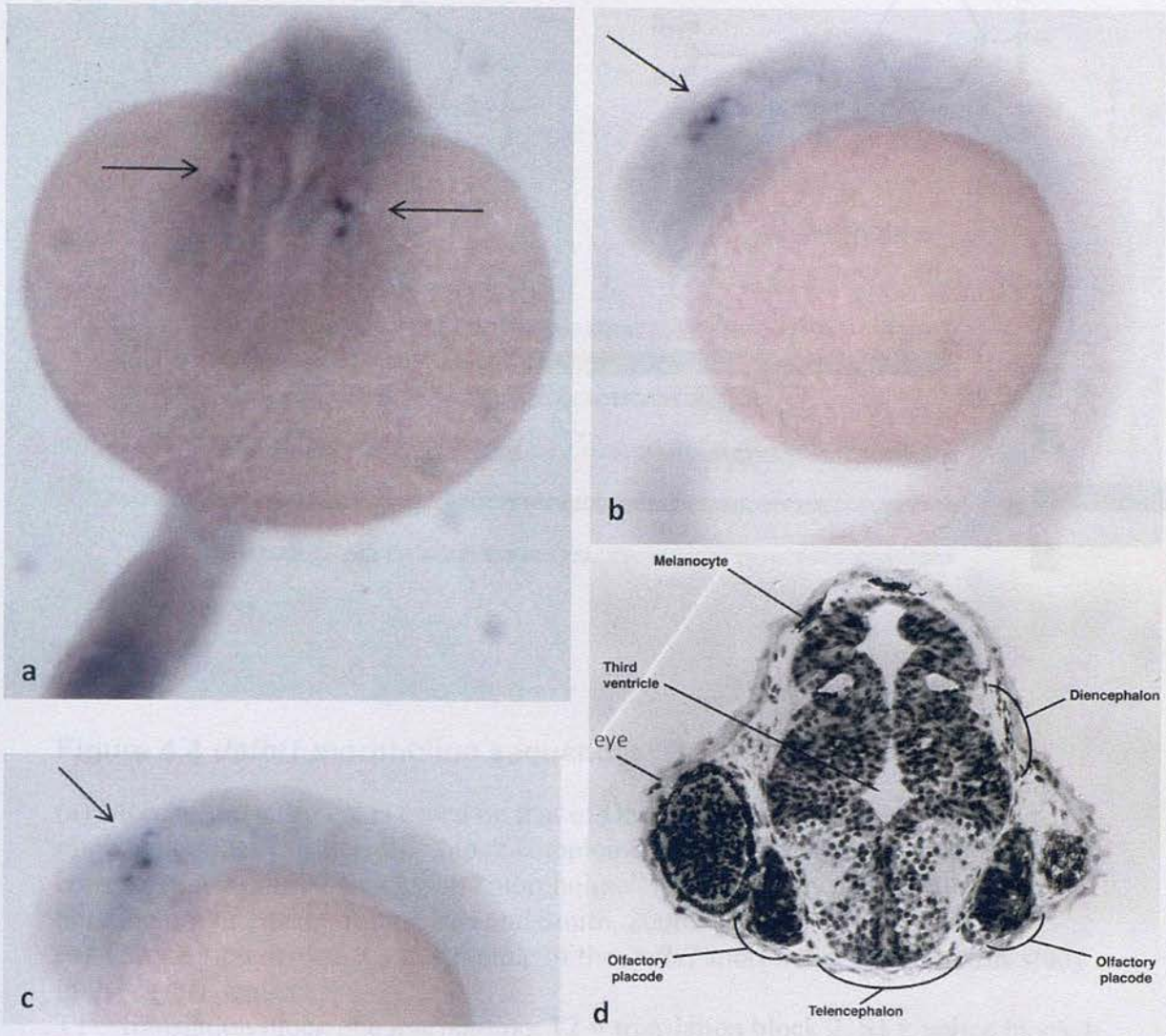


Figure 4.3 Wholemount in situ hybridisation staining for *defbl1* in 24hpf embryos

(a) embryo depicted from dorsal anterior view. Arrows point to two areas of *defbl1* staining. The contrast in this picture has been digitally altered to better reflect observations made "by eye".
(b)/(c) *defbl1* staining viewed in lateral orientation
(d) Depicts a section through the zebrafish embryonic brain at 48hpf (dorsal view), showing the location of the diencephalon with respect to other neural tissues. (taken from http://zfin.org Anatomy Database, image: ZDB_IMAGE_100806-23)

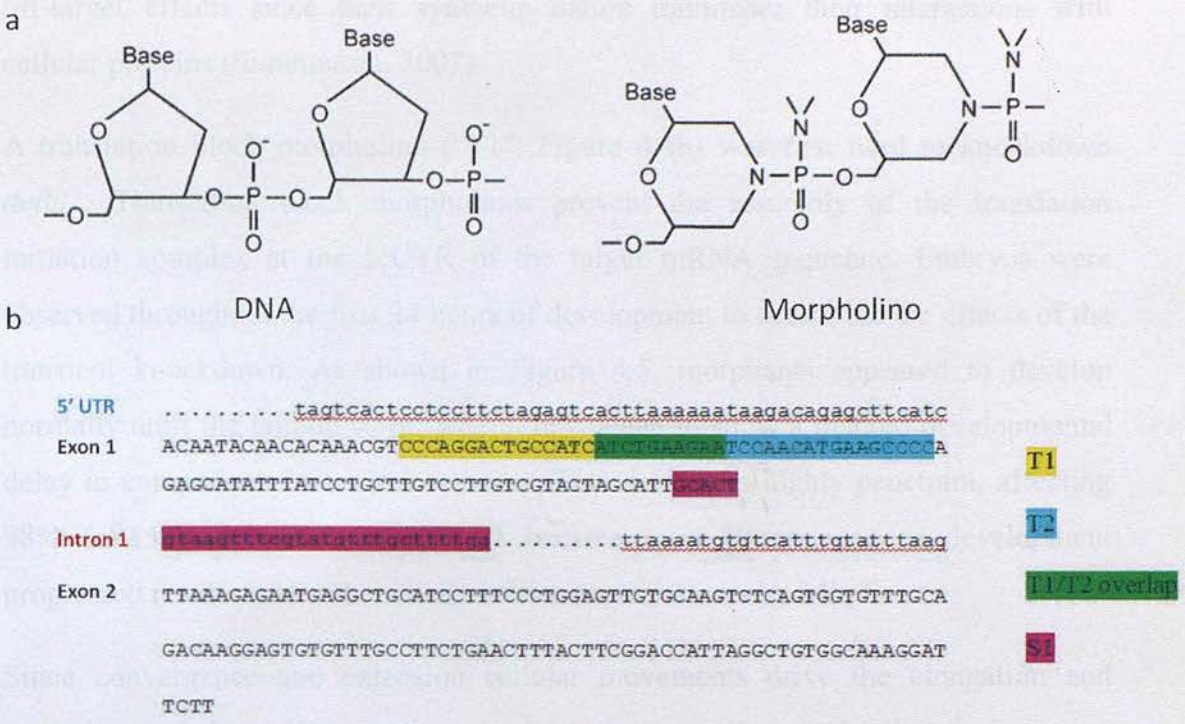


Figure 4.4 *defbl1* morpholino sequences

(a) Morpholino structure is based on that of DNA and is such that morpholino oligonucleotides can sterically block complementary DNA sequences. Morpholinos consists of a modified six-carbon "morpholino" ring linked by phosphodiamidate bonding. Picture taken from Eisen and Smith, 2008.
(b) This section depicts the positioning of the *defbl1* morpholinos used in this study in the *defbl1* sequence.
T1 = translation block one morpholino, T2 = translation block 2, S1 = splice block 1, T1/T2 = area where the T1 and T2 morpholinos overlap.

off-target effects since their synthetic nature minimises their interactions with cellular proteins (Summerton, 2007).

A translation block morpholino ("T1", Figure 4.4b) was first used to knockdown *defbl1*. Translation block morpholinos prevent the assembly of the translation initiation complex at the 5'UTR of the target mRNA sequence. Embryos were observed throughout the first 24 hours of development to determine the effects of the transient knockdown. As shown in Figure 4.5, morphants appeared to develop normally until the tailbud stage, where they began to show a marked developmental delay in comparison to control embryos. This delay was highly penetrant, affecting 98% (n=152) morphant embryos. It became more pronounced as development progressed resulting in a shortened anterior-posterior axis by 24hpf.

Since convergence and extension cellular movements drive the elongation and narrowing of the embryonic anterior-posterior axis during development, it was reasoned that the observed delay might be caused by a defect in these morphogenic cell movements. To quantify this delay, the distance between the head and tail of the morphants was measured, to gauge any difference in anterior-posterior axis elongation. Measurements were taken at both 13hpf and 14hpf, and it was found that the axis elongation was significantly decreased in morphants (compared to controls) at 14hpf (2-4 somites) (Figure 4.6). The shortened anterior-posterior axis can be observed clearly in 24hpf morphants (Figure 4.7a,b).

To gain further insight into the convergence-extension defect, wholemount in situ hybridisation (WISH) was carried out to visualise the location of a number of key cell movement markers. Embryos were firstly stained for *hgg1* and *dlx3*. *hgg1*, a marker of the anterior-posterior axis and *dlx3*, a convergence marker which localises to the edge of the neural plate (either side of the developing notochord; Schulte-Merker *et al.*, 1994) were stained in 14hpf (4-somite) control and morphant embryos. In the *defbl1* morphants, *hgg1* showed more diffuse staining than control embryos, whereas the *dlx3* showed a dramatic change, with staining indicating the neural plate was wider than in wild types. This suggested that convergent cellular movements were retarded in the morphant embryos (Figure 4.8). The movement defect was quantified and found to be statistically significant (Figure 4.9).

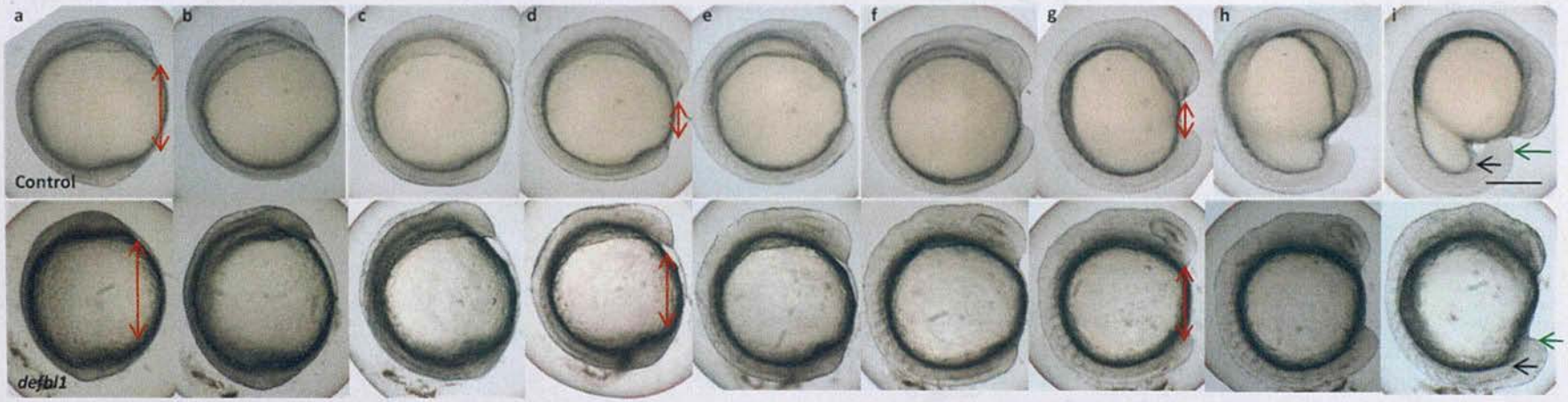
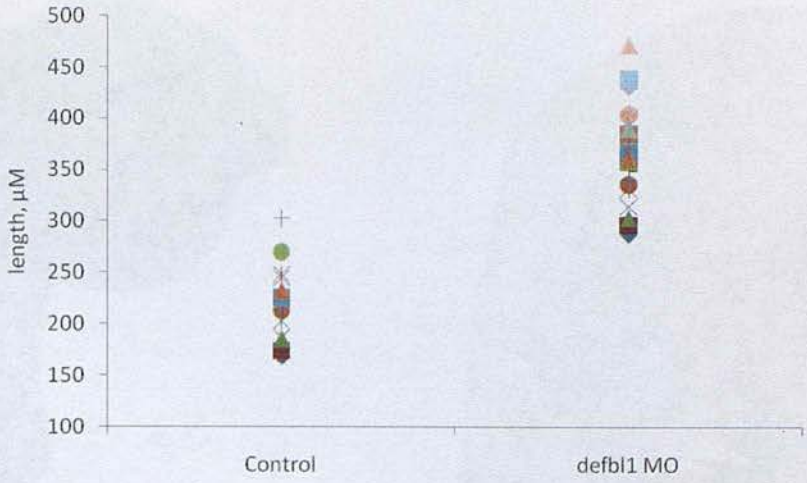


Figure 4.5 Phenotype of the *defbl1* T1 morpholino

Embryos were imaged hourly from **(a)** 13hpf (1-2 somites) to **(i)** 21hpf. The upper panel represents embryos injected with control morpholino and the lower, those injected with *defbl1* "T1" morpholino. Embryos were staged and matched at 8hpf in preparation for the study.

Morphant embryos showed delayed progression of axis elongation, as evidenced by a failure of the lead-edges of the developing head and tail to migrate around the yolk-sac (highlighted in panels (a), (d) and (g) by the double-headed red arrow). In addition, morphants show a lack of early gut elongation (black arrow, panel (i)) and slowed extension of the developing tail (green arrow, panel (i)). Scale bar = 100μm



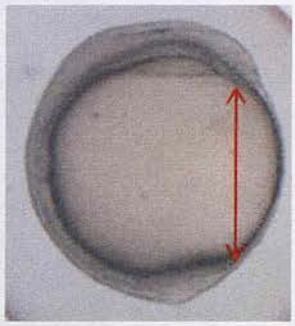


Figure 4.6 Head-tail distance in 14hpf control and *defbl1* morphant embryos

Head-tail distance was measured (µm). The difference between morphants and control-injected was significantly different ($p<0.0005$).

Figure 4.7 The *defbl1* morphant phenotype at 24hpf

This figure presents the *defbl1* morphant phenotype. The upper panel shows a wild type embryo, with a close up image showing brain and eye structure at higher magnification. The lower panel depicts a representative *defbl1* morphant at 24hpf. The anterior-posterior axis is strikingly shortened and the brain shows no compartmentalisation (upper arrow) as seen in the control embryo. The developing eye is also an aberrant shape (lower arrow).
Scale bars = 100μm

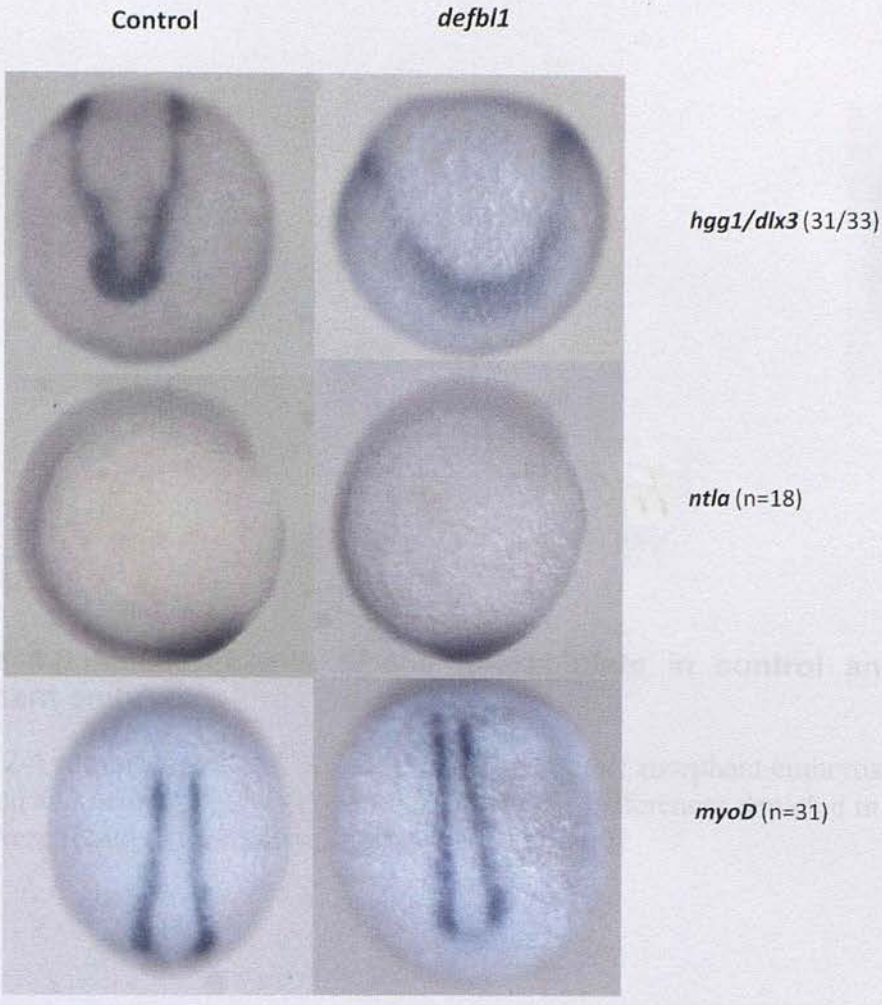


Figure 4.8 Wholemount in situ hybridisation (WISH) on 14hpf *defbl1* morphants

Control–injected and *defbl1* morphant 14hpf (2-4 somite) embryos were stained for a number of developmental markers. The above shows representative pictures of the phenotypes observed. Sample numbers are provided next to gene names. For *hgg1/dlx3* staining, 31/33 stained embryos showed the aberrant phenotype depicted in the upper panel (data pooled from three independent injection sets).

hgg1/dlx3 and *myoD* are orientated to show the anterior-posterior axis, *ntla* embryos are imaged laterally.

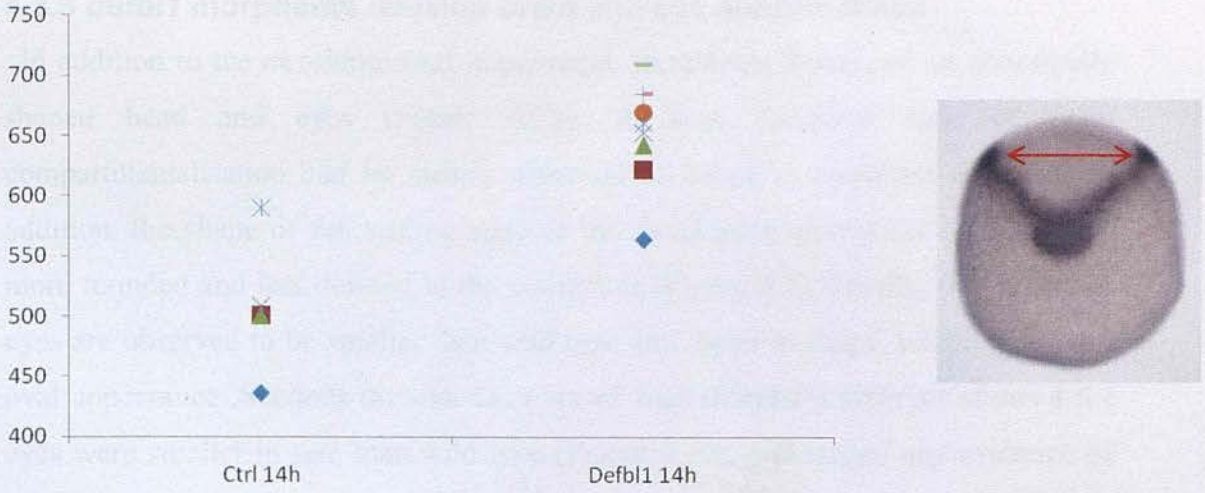


Figure 4.9 Measurements of the neural plate in control and *defbl1* morphant embryos

14hpf (2-4 somite) *hgg1/dlx3* stained control and *defbl1* morphant embryos were collected and neural plate width was measured. The differences depicted in this graph were found to be statistically significant, $p<0.001$.

4.3.3 *defbl1* morphants develop brain and eye abnormalities

In addition to the developmental impairment, morphants developed an abnormally shaped head and eyes (Figure 4.7b). A near complete lack of brain compartmentalisation can be clearly observed at 24hpf in morphant embryos. In addition, the shape of the leading edge of the developing embryonic head appears more rounded and less defined in the morphants (Figure 4.7). Finally, the morphant eyes are observed to be smaller than wild type and differ in shape, adopting a more oval appearance. Sections through the eyes of 4dpf morphant embryos showed the eyes were smaller in size than wild type (Figure 4.10), and lacked any evidence of differentiation to form the stratified layers observed in wild type embryos. This indicates a suspension of optical development prior to 48hpf when the layers first differentiate. The morphant lens, while remaining a similar size to the wild type, also contained nuclei (Figure 4.10), not seen in wild type embryos. No normal eyes were observed in morphant sectioned eyes slides (n=22).

4.3.4 Verifying the *defbl1* morphant phenotype

Non-specific "off-target" effects are a possibility when using any knockdown technique. In zebrafish, these effects can include developmental delay and widespread neural necrosis and, in many cases, have been attributed to p53 activation (Danilova *et al.*, 2010). To ensure that the observed *defbl1* morphant phenotype was not due to non-specific p53 activation, *defbl1* "T1" morpholino was injected into $p53^{M214K/M214K}$ embryos (these embryos encode a non-functional, dominant negative p53). Embryos were observed at 24hpf and 48hpf and still maintained the same mutant phenotype as described previously (data not shown), so *p53* activation was ruled out as a primary cause of the mutant phenotype.

In order to further confirm the specificity of the observed defbl1 phenotype, a second translation block morpholino, "T2" was designed (Figure 4.4b) and injected into embryos. 80.9% injected embryos (119/147) were observed to show a developmental delay beginning at 13-14hpf, along with abnormal brain and eye structure at 24hpf (Figure 4.11), replicating the "T1" morpholino phenotype. However, since "T2" has a 10 base overlap with "T1" and therefore is not unique, the morphant phenotype was

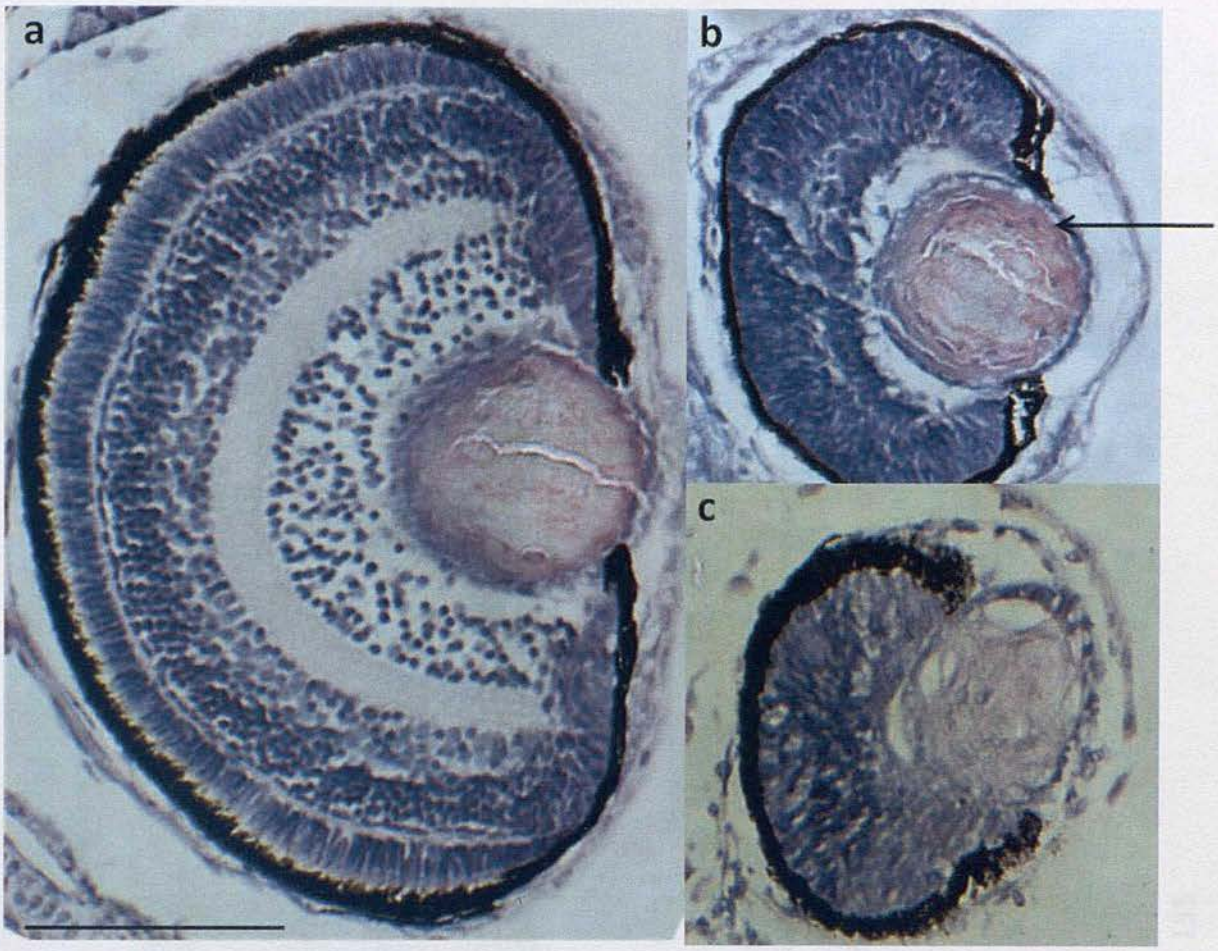


Figure 4.10 Sections through eyes of *defbl1* morphants, compared to controls

(a) shows haematoxylin and eosin staining of sections through a control morpholino-injected embryonic eye (4dpf)
(b),(c) a representative image of a typical *defbl1* morphant eye at 4dpf (n=22). Arrow points out the presence of a persistent nucleus in the lens
Images captured at x20 magnification, scale bar = 100μm.

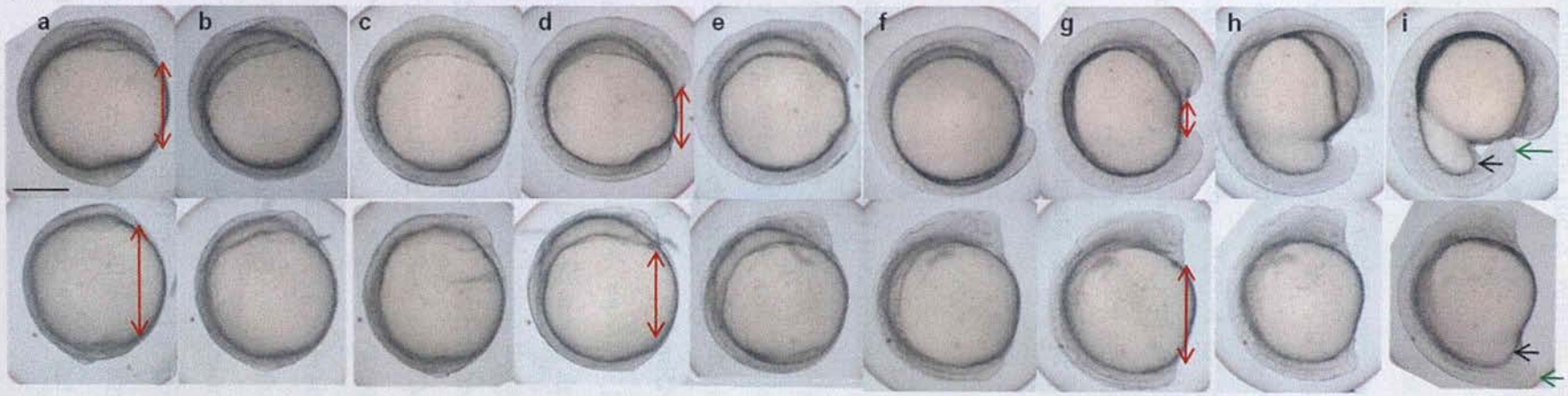


Figure 4.11 Phenotype of the *defbl1* T2 morpholino

Embryos were imaged hourly from **(a)** 13hpf (1-2 somites) to **(i)** 21hpf. The upper panel represents embryos injected with control morpholino and the lower, those injected with *defbl1* "T2" morpholino. Embryos were staged and matched at 8hpf in preparation for the study.

As with *defbl1* "T1" morphants, *defbl1* "T2" morphants showed delayed progression of axis elongation, as evidenced by a failure of the lead-edges of the developing head and tail to migrate around the yolk-sac (highlighted in panels (a), (d) and (g) by the double-headed red arrow). In addition, morphants show a lack of early gut elongation (black arrow, panel (i)) and slowed extension of the developing tail (green arrow, panel (i)).

also replicated using a splice-block morpholino, "S1". The splice-block morpholino was designed to bind the 5' regions of intron 1 and its adjacent exon to result in intron inclusion and a transcript containing a premature stop codon (Figure 4.4b, 4.12a). As observed with morpholinos "T1" and "T2", 71% "S1" morphants (n=112) showed a slowing of development, resulting in a reduced anterior-posterior axis at 22hpf (Figure 4.12b). A higher concentration of "S1" morpholino was required to achieve the same phenotype as "T1", and the penetrance was reduced (71% (80/112) morphants showed phenotype), however aberrantly spliced *defbl1* mRNA was detected in RNA from affected morphant embryos (Figure 4.12c). Sequencing revealed that the "S1" morpholino allowed for the inclusion of both introns of the *defbl1* transcript (due to sequence similarity at the 5' end of introns 1 and 2) and the presence of this aberrant transcript corresponded with the presence of mutant phenotype.

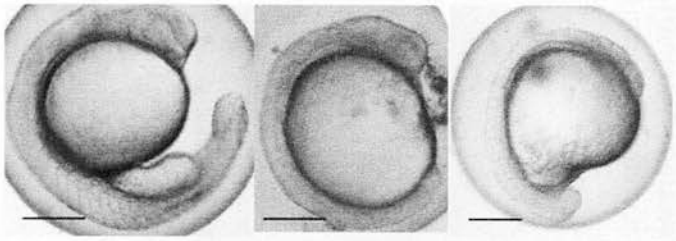
4.3.5 Rescue of *defbl1* morphants

In order to prove the specificity of the d*efbl1* "T1" morpholino, morphants were rescued using *defbl1* mRNA. The "T1" morpholino was chosen to rescue due to the severity and high penetrance of the phenotype. Originally, mutations were introduced into the 5'UTR of the synthesised mRNA in order to prevent the morpholino from binding it. However, these mutants proved difficult to transcribe in adequate concentrations for injection, so it was decided an excess volume of wild type mRNA should have the effect of diluting the morpholino phenotype. As shown in Figure 4.13, the co-injection of "T1" morpholino and d*efbl1* mRNA resulted in the recovery of a normal phenotype for 26.6% (25/94) injected embryos. In addition, a "dilution effect" was observed, with 22.3% (21/94) (embryos exhibiting a "mild" phenotype (classed as having either an eye or brain phenotype, not both), leaving only 28.7% (27/94) co-injected embryos showing the full *defbl1* morphant phenotype (vs 89.1% (114/128) injected with morpholino alone). In addition, sections through the eyes of rescue morphants at 4dpf revealed a similar statistic (Figure 4.14); while 25% (7/28) co-injected embryos exhibited a severe morphant phenotype, a further 46.4% (13/28) exhibited a "mild" phenotype, where the eyes maintained their reduced size but

a

Morpholino	Translated sequence	Expected band, bp
Control/T1/T2	MKPQSIFILLVLVVLALHCKENEAASFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFLCCVSHFLStop	181
S1 (intron 1)	MKPQSIFILLVLVVLALHCKFRMFAFGDISVLELCStop	409
S1 (intron 2)	MKPQSIFILLVLVVLALHCKENEAASFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFLStop	275
S1 (intron 1+2)	MKPQSIFILLVLVVLALHCKFRMFAFGDISVLELCStop	503

b



c

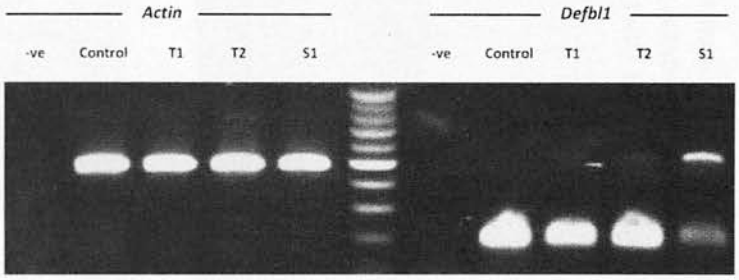


Figure 4.12 *defbl1* S1 morpholino phenotype

(a) This table shows the predicted translated peptide sequences following embryonic injection with *defbl1* S1 morpholino. The far left column details the morpholino details and which introns is targeted. Expected diagnostic RT-PCR band sizes are depicted in the far right column.

(b) The *defbl1* S1 phenotype is similar to the T1 phenotype. Pictures taken at 22hpf, scale bar = 100μm. S1 morphant picture is a representative picture based on three independent experimental repeats containing a combined total of 112 embryos.

(c) Diagnostic RT-PCR to detect *defbl1* transcript size changes. Control, T1 and T2 embryos have a band at 181bp, indicating wild type splicing. cDNA from S1-injected morphants have an aberrant transcript size of 503bp, indicating inclusion of both introns. For additional clarity, the identity of bands from these gels were confirmed by sequencing (data not shown)

Injection	Normal (%)	Mild (%)	Severe (%)	Toxic (%)
defbl1 T1 MO	2.3	1.6	89.1	7
defbl1 mRNA	57.9	0	7.5	34.6
T1 MO + mRNA	26.6	22.3	28.7	22.3

Figure 4.13 *defbl1* T1 morphant rescue

Embryos were injected with either 15μM T1 morpholino (n=128), 20ng/μl RNA (n=107) or a mixture of both (n=94), and phenotypes at 48hpf were recorded. Morphant phenotype were categorised as "severe" (as described previously) or "mild" (showing abnormal eyes or an abnormal brain, but not both). "Toxic" denotes animals that had died by 24hpf. n numbers are based on the number of embryos surviving the injection process by 6hpf.

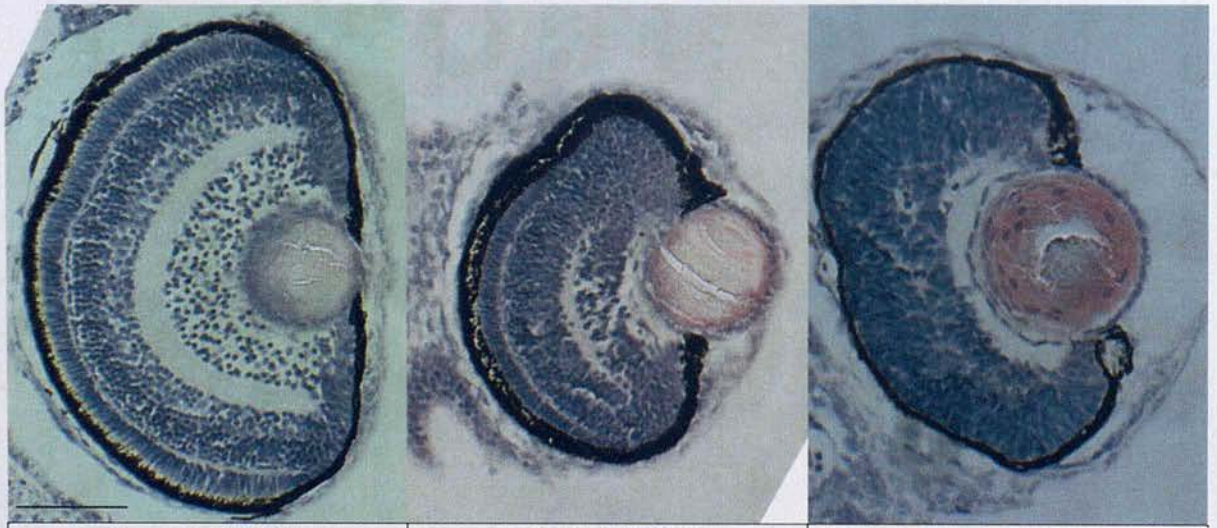


Figure 4.14 Eye phenotype in embryos co-injected with *defbl1* morpholino and *defbl1* mRNA

Eyes from 4dpf embryos co-injected with *defbl1* morpholino and mRNA were sectioned and haemoxylin and eosin stained to reveal any rescue of the morphant eye phenotype shown in Figure 4.10.
Scale bar = 100μm and pictures were captured at x20 magnification.

showed some evidence of differentiation to form stratified layers, and had a lens free from nuclei. In addition, 28.6% (8/28) embryos showed normal eye development, indistinguishable from wild type. This indicates that co-injection of *defbl1* morpholino with *defbl1* mRNA is able to rescue both the developmental and optical defects previously described.

4.4 MICROARRAY ANALYSIS OF *DEFBL1* MORPHANTS

4.4.1 Microarray analysis of *defbl1* morphants compared to controls

In the absence of an antibody to Defbl1, microarray analysis was performed on morphant embryos in order to gain insight into the global gene expression changes resulting from *defbl1* knockdown. RNA was harvested from morphant and control-injected embryos at 12.5hpf, just after the first d*efbl1* transcripts can be detected by RT-PCR. Samples were sent for hybridisation using Agilent Whole Zebrafish Genome Oligo Microarrays (Miltenyi Biotec GmbH) on two independent biological repeats.

Analysis of the data revealed 6553 statistically significant ($p<0.05$) differentially expressed genes between control and morphant embryos. This included an up-regulation of 3164 genes and a further down-regulation of 3389 genes. In order to assess any patterns in these genes, the Database for Annotation Visualization and Integrated Discovery (DAVID) (Huang *et al.*, 2009a, Huang *et al.*, 2009b) was used to group the genes according to their area of expression in the zebrafish (ZFIN_ANATOMY search, Table 4.1). Concordant with the abnormal brain and eye structures seen in *defbl1* morphants, a large proportion of the differentially expressed genes are located in the embryonic eye and brain. Moreover, the structure associated with the highest grouped percentage of differentially expressed genes reported was the retina (Table 4.1). Table 4.1 shows a heavy representation of neural structures, including the diencephalon as the structure showing the most significantly affected subset of genes. Key developmental structures are also highlighted, including the neural crest, corresponding to the developmental defects observed in the *defbl1* morphants. The highlighting of the brain, eye and developmental tissues is in accordance with the observed *defbl1* morphant phenotype.

Anatomical Structure	% genes	p value	Anatomical Structure	% genes	p value
retina	8.29	7.41E-07	neural plate	4.39	1.02E-10
optic tectum	6.08	6.55E-06	pronephric duct	3.82	0.001968
optic vesicle	3.54	5.43E-08	YSL	3.49	0.002863
lens	2.86	0.003204	tegmentum	3.37	6.48E-04
lens vesicle	2.56	6.79E-04	epiphysis	3.16	4.79E-05
retinal ganglion cell layer	2.24	0.001553	olfactory placode	3.04	9.35E-06
retinal inner nuclear layer	1.66	7.24E-04	periderm	3.01	6.17E-06
presumptive neural retina	0.9	0.00398	ventral mesoderm	2.74	7.89E-07
optic primordium	0.83	0.006977	ventricular zone	2.56	4.35E-05
hindbrain	7.84	1.64E-06	adaxial cell	2.46	3.99E-04
spinal cord	6.86	7.55E-07	otic placode	2.34	7.11E-04
diencephalon	6.65	2.59E-11	neural crest	1.73	1.75E-06
midbrain	6.23	3.70E-05	trigeminal placode	1.68	6.46E-06
central nervous system	6.05	1.03E-06	heart tube	1.58	6.89E-04
telencephalon	5.4	2.48E-07	margin	1.53	0.001542
neural tube	5.22	1.39E-09	heart rudiment	1.41	0.001437
forebrain	3.77	5.81E-05	pectoral fin bud	1.33	0.002769
neuron	3.62	1.81E-06	forerunner cell group	0.88	0.00382
cranial ganglion	3.06	5.52E-06	lateral plate mesoderm	0.8	0.005669
alar plate midbrain region	2.69	0.004994	hypaxial myotome region	0.8	0.006734
basal plate midbrain region	2.24	3.53E-05	pancreas primordium	0.65	0.003134
head	1.71	4.25E-04	anterior lateral plate mesoderm	0.48	6.75E-04
midbrain hindbrain boundary	1.71	0.005164	posterior lateral plate mesoderm	0.45	0.002044
rhombomere	1.63	5.63E-05	gut	3.89	9.83E-05
neural rod	1.58	6.89E-04	pectoral fin	3.77	0.001083
hindbrain neural plate	1.53	4.63E-06	pharyngeal arch	3.74	9.34E-10
midbrain neural keel	1.33	2.73E-06	heart	3.21	9.16E-04
primary neuron	0.83	1.93E-04	epidermis	2.86	4.89E-06
rhombomere 5	0.68	8.23E-04	ovary	2.06	2.60E-04
hindbrain neural keel	0.63	1.15E-04	musculature system	1.93	2.12E-04
midbrain neural rod	0.55	5.55E-04	cleithrum	0.6	0.005054
neuroectoderm	0.53	0.004618	slow muscle cell	0.43	7.28E-04
rhombomere 3	0.5	0.001711	caudal fin	0.43	0.009699
presumptive rhombomere 3	0.23	0.001927	trunk vasculature	0.38	0.002525
somite	7.63	1.87E-08	pharyngeal pouch	0.35	0.001129
otic vesicle	5.02	1.24E-05	fast muscle cell	0.35	0.001756
myotome	4.8	1.03E-05	opercle	0.28	0.009753
notochord	4.42	0.006475	angioblastic mesenchymal cell	0.23	0.003659

Table 4.1 Clustering of genes differentially expressed in *defbl1* morphants with respect to control embryos (table shown on previous page)

"DAVID" software (Huang *et al.*, 2009a; Huang *et al.*, 2009b) was used to cluster genes according to their anatomical location (as described by the Zebrafish Information Network, ZFIN). Only structures significantly enriched ($p<0.01$) were included.

Structures highlighted in yellow represent genes predominantly expressed in the adult or developing eye; structures in blue are associated with the adult or developing brain and neural structures; structures in pink are associated with developmental structures not found in the adult and purple structures are those which cannot be grouped into any of the above categories.

To investigate further any enrichment ($p<0.01$) in the differentially expressed genes, the group was analysed according to biological function gene ontology (GO) terms. The search revealed a wide variety of biological processes affected (Table 4.2) with processes ranging from vital cellular processes such as transcription and protein signalling, to more specific growth and development pathways. In particular, genes affecting embryonic morphogenesis and organogenesis were enriched, along with those involved in cell motion and migration.

The GO term analysis revealed a number of pathways which may be key in the pathology of the *defbl1* morphant phenotype and these were not all predicted from based on the *defbl1* morphant knockdown. For example, transcription and regulation of transcription were highly enriched, suggesting that *defbl1* might have a role which directly or indirectly interacts with transcription factors. As with the anatomy clustering, developmental processes were also enriched, with regulation of neurogenesis and embryonic morphogenesis being identified as being significantly enriched. Interestingly, regulation of cell migration and regulation of cell motion were both detected, which may be illustrative of the convergence defects observed in the *defbl1* morphants.

Although these analyses identified the gene expression locations and processes which are enriched within the microarray, the results obtained were wide-ranging and there was often overlap between clustering terms (for example "regulation of growth", "regulation of cell growth", Table 4.2). To narrow the processes affected, the differentially expressed gene group was analysed again, grouping genes according to a set of keywords. Each keyword covers a number of GO terms, therefore the resulting number of categories were narrowed, which would possibly allow for more trends to be observed. The analysis (Table 4.3) revealed a number of processes observed in the GO term analysis; again, changes in expression of genes involved in transcription and regulation of transcription were highly enriched as were the categories of "developmental proteins" and homeobox genes. Interestingly, this analysis also detected a significant enhancement in the Wnt signalling pathway (Table 4.3). The Wnt-PCP signalling pathway has a crucial role in the mediation of convergence and extension movements during gastrulation (reviewed in Roszko *et*

GO Term	%	p value	GO term
regulation of transcription	8.51	3.76E-11	GO:0045449
regulation of transcription, DNA-dependent	6.96	7.93E-14	GO:0006355
transcription	3.82	3.16E-04	GO:0006350
regulation of RNA metabolic process	7.01	1.05E-13	GO:0051252
carbohydrate catabolic process	0.7	0.0022	GO:0016052
alcohol catabolic process	0.63	6.94E-04	GO:0046164
cellular carbohydrate catabolic process	0.63	6.94E-04	GO:0044275
glucose metabolic process	0.63	0.0059	GO:0006006
glucose catabolic process	0.55	0.0027	GO:0006007
hexose catabolic process	0.55	0.0027	GO:0019320
monosaccharide catabolic process	0.55	0.0027	GO:0046365
glycolysis	0.5	0.0016	GO:0006096
pattern specification process	2.03	2.13E-04	GO:0007389
embryonic morphogenesis	1.83	0.0011	GO:0048598
regionalization	1.43	0.0073	GO:0003002
cellular component morphogenesis	1.36	5.74E-04	GO:0032989
embryonic organ development	1.31	6.94E-04	GO:0048568
cell morphogenesis	1.08	0.0046	GO:0000902
cell part morphogenesis	0.88	0.0035	GO:0032990
cell projection morphogenesis	0.85	0.0038	GO:0048858
embryonic organ morphogenesis	0.75	0.0083	GO:0048562
muscle cell differentiation	0.6	0.0051	GO:0042692
cell fate commitment	0.58	0.0014	GO:0045165
striated muscle cell differentiation	0.58	0.0055	GO:0051146
muscle cell development	0.5	0.003	GO:0055001
regulation of neurogenesis	0.48	7.72E-04	GO:0050767
regulation of nervous system development	0.48	7.72E-04	GO:0051960
striated muscle cell development	0.48	0.0043	GO:0055002
regulation of cell development	0.48	0.0016	GO:0060284
notochord development	0.4	0.0092	GO:0030903
regulation of growth	0.63	3.55E-05	GO:0040008
regulation of cell growth	0.55	1.09E-04	GO:0001558
regulation of cell size	0.4	0.0092	GO:0008361
negative regulation of growth	0.2	0.0045	GO:0045926
regulation of cell motion	0.28	0.0045	GO:0051270
regulation of cell migration	0.28	0.0045	GO:0030334
cell projection organization	1	0.0044	GO:0030030
melanosome localization	0.2	0.0086	GO:0032400
pigment granule transport	0.2	0.0086	GO:0051904

Table 4.2 Clustering of differentially expressed genes by biological function gene ontology

"DAVID" software (Huang *et al.*, 2009a; Huang *et al.*, 2009b) was used to cluster genes according to their biological function GO terms. Only structures significantly enriched ($p<0.01$) were included. Processes highlighted in pink are related to transcription, processes in blue related to catabolism, processes in yellow relate to embryonic development (in various tissues) and processes highlight in green relate to cell growth.

Term	% genes	p value
dna-binding	6.33	9.18E-19
Homeobox	2.89	3.88E-14
developmental protein	3.62	4.93E-14
nucleus	8.74	1.09E-08
Transcription	4.09	6.91E-08
transcription regulation	4.12	9.48E-08
glycolysis	0.43	2.75E-04
DNA binding	0.43	4.39E-04
signal	2.06	0.0034
glycoprotein	2.31	0.0092
egf-like domain	0.98	0.0098
wnt signaling pathway	0.63	0.0099

Table 4.3 Genes differentially expressed in the microarray are sorted according to PIR_KEYWORDS

"DAVID" software (Huang *et al.*, 2009a; Huang *et al.*, 2009b) was used to cluster genes according to the PIR_KEYWORDS database. Terms enhanced at a probability of $p<0.01$ are listed.

al., 2009). It is, therefore, interesting that the genes involved in this process are significantly differentially expressed in the *defbl1* morphants, compared to the control embryos. Figure 4.15 illustrates the Wnt pathway and the genes identified as being differentially expressed in the microarray.

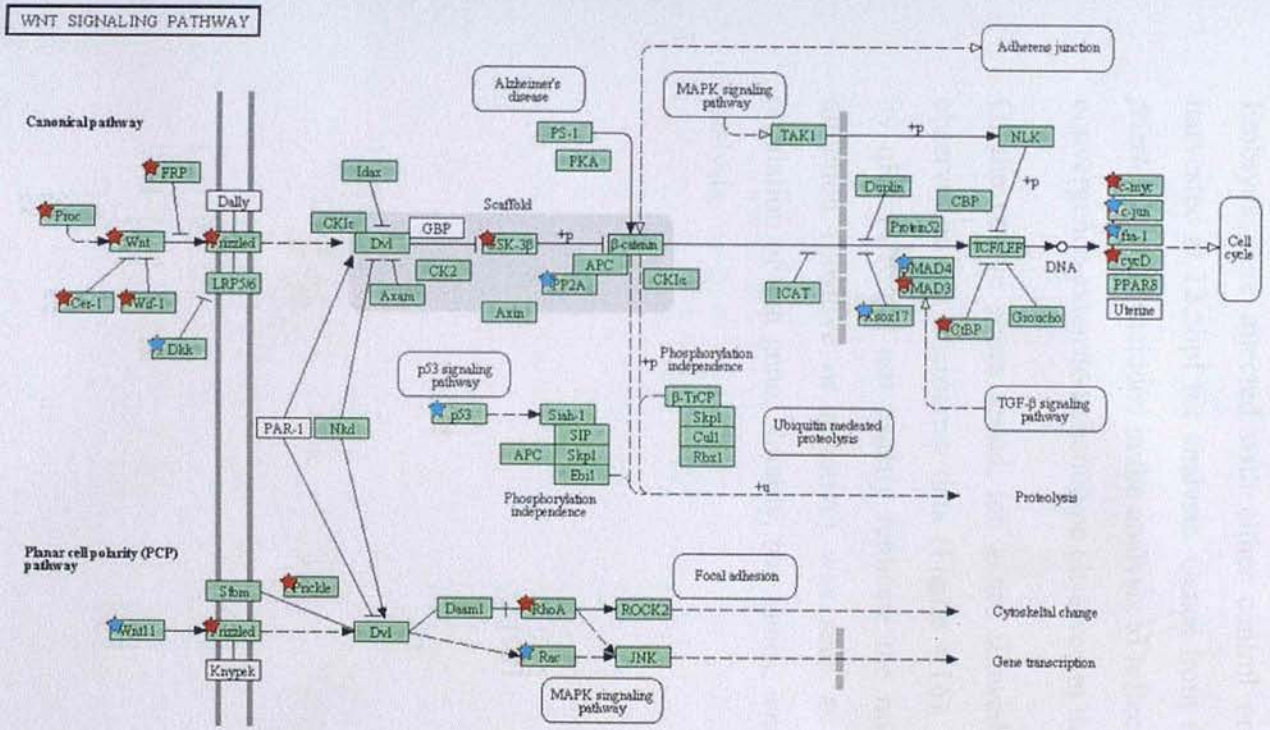


Figure 4.15 The Wnt-PCP pathway is enriched in the *defbl1* morphant microarray analysis

DAVID software was used to map affected genes in the array to the Wnt-PCP signalling pathway (using the KEGG_PATHWAY functional annotation function). Red stars denote genes down-regulated in the array, blue stars denote up-regulated genes.

4.4.2 Validation of microarray data using quantitative RT-PCR

In order to begin to validate the microarray data, a panel of genes were selected for analysis using qRT-PCR. The panel of genes chosen included some from the Wnt-Pcp pathway (as representatives of a pathway of interest), as well as other random genes found to be significantly altered between control and morphant embryos. A selection of genes outwith the Wnt-Pcp pathway was included in this analysis to broaden the range of genes tested so as not to introduce bias into this testing.

Embryos were injected with either control or *defbl1* morpholino and RNA was harvested at 12.5hpf for analysis. Genes from the Wnt-PCP pathway (*wnt11*, *wnt8*, *frizzled2*) were included in the analysis to reflect the relevance of the pathway to the convergence-extension phenotype observed in the *defbl1* morphants.

Of the twelve genes tested, ten genes showed expression trends similar to those observed in the microarray data (Figure 4.16). Although the fold-changes obtained by qRT-PCR did not exactly replicate the microarray data, a trend in the same direction (positive or negative) was seen as verification of the up- or down-regulation of that gene. Notably, two genes, *wnt8* and *p53* were not verified by this analysis.

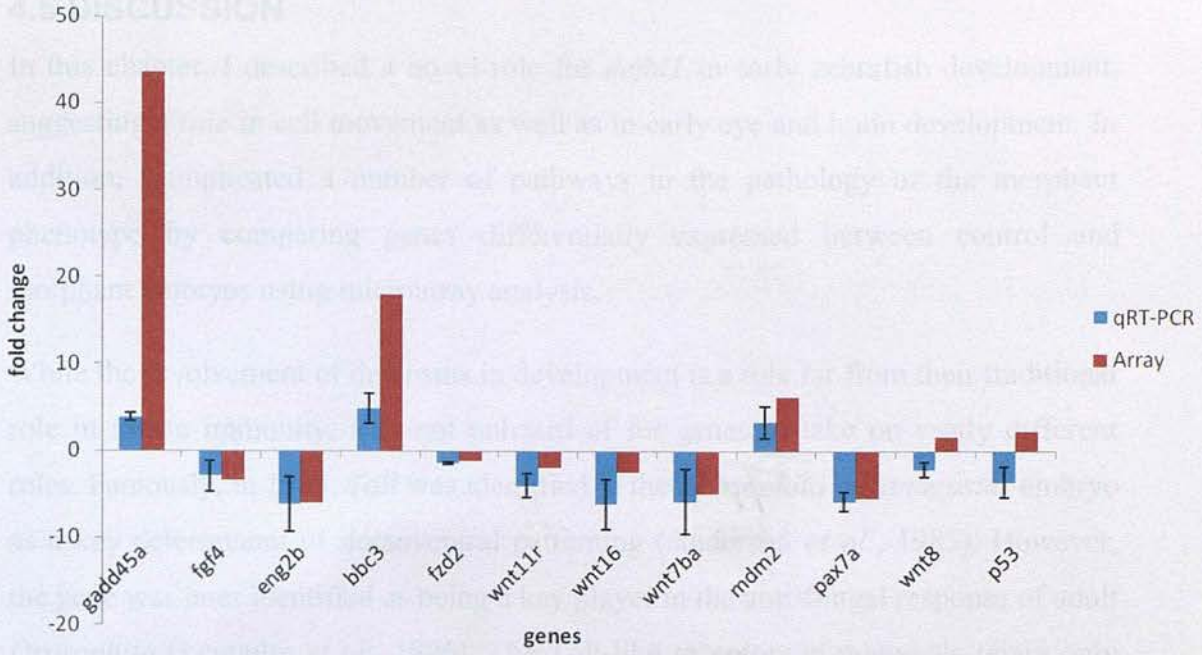


Figure 4.16 qRT-PCR validation of microarray data

Quantitative RT-PCR was used to validate the expression changes of twelve genes identified as being differentially expressed in the microarray analysis. Data is based on three independent repeats carried out on three biological samples. Fold changes are measured relative to control embryos.

4.5 DISCUSSION

In this chapter, I described a novel role for *defbl1* in early zebrafish development, suggesting a role in cell movement as well as in early eye and brain development. In addition, I implicated a number of pathways in the pathology of the morphant phenotype by comparing genes differentially expressed between control and morphant embryos using microarray analysis.

While the involvement of defensins in development is a role far from their traditional role in innate immunity, it is not unheard of for genes to take on vastly different roles. Famously, in 1985, *Toll* was identified in the *Drosophila melanogaster* embryo as a key determinant of dorsoventral patterning (Anderson *et al.*, 1985). However, the gene was later identified as being a key player in the anti-fungal response of adult *Drosophila* (Lemaitre *et al.*, 1996). The Toll-like receptors in mammals retain only the immunological role.

There have been recent reports which also link the mammalian defensins to varied developmental processes. It has been reported that a knockdown of epididymis-specific *Defb15* in *R.norvegicus* causes decreased adult fertility and developmental failure in offspring (Zhao *et al.*, 2011). Murine *Defb19* has also been implicated in testis development (Menke and Page, 2002). Interestingly, the Eurexpress database (a transcriptome atlas for the mouse embryo) also shows specific expression of murine *Defb50* in the developing brain and spinal cord at E14.5 (Richardson *et al.*, 2009).

4.5.1 The *defbl1* morphant phenotype

The d*efbl1* "T1" morphant phenotype was explored by imaging morphant embryos throughout the first 24 hours of development to visualise the defects caused by transient *defbl1* knockdown. The observed convergence extension cellular movement delay was quantified by measuring the anterior-posterior axis of morphants, as well as by measuring the neural plate width in embryos stained using WISH for *hgg1* and *dlx3*.

In order to prove the specificity of the "T1" translation block morpholino, it was shown that its phenotype is replicable with two additional morpholinos "T2" and the

splice-block morpholino "S1". The splice block morpholino was selected because the morphant phenotype could be correlated to an aberrantly spliced *defbl1* mRNA transcript, detectable by RT-PCR. This helped to discount the notion that the observed phenotype might be attributable to non-specific interactions, particularly since the "T1" and "T2" morpholinos shared a 10 base overlap. However, it should be noted that the presence of aberrant *defbl1* transcript does not preclude the embryo from also suffering an additional non-specific response.

The *defbl1* morphant phenotype was also shown to be partially rescued upon the introduction of full length wild type *defbl1* mRNA. While the observed rescue was robust, showing recovery of both the developmental and eye and brain phenotypes, 28.7% co-injected embryos did not rescue. However, this observation was somewhat expected due to the wild type nature of the mRNA. The morpholino would have been able to bind and silence this mRNA (in addition to the endogenous transcripts), so the rescue depended on a "flooding" effect, whereby excess wild type transcript was introduced to provide larger volumes of unbound transcript which could be translated appropriately. A mutated mRNA transcript would have avoided morpholino binding and would therefore have been preferable and may have produced a better rescue. However, a PCR fragment containing full length *defbl1* with introduced mutations in the 5'UTR was not compatible with translation (data not shown), indicating that the mutations interfered with the translation process.

It would be interesting to observe whether the Defbl1 peptide is capable of producing a better rescue than *defbl1* mRNA. Although this was attempted (data not shown), no rescue was observed using the synthetic, unfolded Defbl1 peptide described in Chapter 3. However, this was a mature peptide (missing its signal sequence) and therefore may not have been secreted, and was also unfolded which may have led to a loss of functionality.

The work described in this chapter suggests that *defbl1* has multiple roles dependent on the developmental stage of the zebrafish. Zou *et al.*, 2007 showed that *defbl1* is expressed in a number of adult tissues where its role would presumably differ from the embryonic role in convergence extension. In addition, Oehlers *et al*, 2011 showed specific expression of *defbl1* in the post 24hpf embryonic and adult gut as well as

strong expression in the swim bladder. This work led to the hypothesis that Defbl1 is important in antimicrobial defence within these organs. This work would support the notion that the gene takes on different functions at different stages.

The specific WISH expression pattern showing *defbl1* in the embryonic head at 24hpf suggests that the gene can have tissue-specific functionality. However, it should be noted that at 24hpf, *defbl1* expression may not be limited to the head and it is likely that instead it shows low level expression across the embryo. This is supported by the observation that no WISH staining pattern could be determined at 13hpf (data not shown), when the mRNA is still detectable by RT-PCR. The detection of *defbl1* transcripts in the early embryo was not replicated in the Oehlers *et al.*, 2011 study, however this data was not shown or expanded upon. It is possible that since this study was focused on antimicrobial activity and expression in the gut, that earlier expression analyses were not prioritised. Due to the time at which the study was published, there was not sufficient time for the WISH and RT-PCR analysis described in this study to be repeated with the same primers and probes described in Oehlers *et al.*, 2011. However, performing these analyses in the future may provide insight into the reported discrepancies. In addition, obtaining a Defbl1 antibody would also provide insight into whether the observations of *defbl1* mRNA transcripts correspond to the protein levels.

The notion that *defbl1* has multiple roles highlights the lack of clarity as to whether the eye and brain phenotype observed in *defbl1* morphants are related to the earlier developmental delay. It is possible that the defect in convergence extension movements brings about defects in brain and eye development by affecting the migration of neuronal and optical precursor cells from the ectoderm. However, it is equally possible that *defbl1* has additional roles in eye and brain development. It has previously been shown that macrophages play a crucial role in tissue remodelling in the developing mouse eye (Lang and Bishop, 1993). The macrophages are reported to be responsible for the regression of two ocular layers normally only transiently present in early optical structures. Since *defbl1* morphant eyes never undergo remodelling to develop stratified cell layers, it is possible that macrophages might play a role in this phenotype. It has been shown that macrophages invade the

cephalic mesenchyme of developing zebrafish embryos and migrate into the tissues of the developing brain and retina (Herbomel *et al.*, 2001), although it is unknown what role they fulfil. This, coupled with the finding that mammalian defensins are chemoattractants for macrophages (Soruri *et al.*, 2007) suggests that the interaction of *defbl1* with macrophages in the developing eye and brain is a candidate process which may, when disrupted, cause a phenotype independent of the earlier observed developmental delay. This role is supported by the fact that primitive macrophages are first observed in the developing embryo from 14-16hpf, far later than the first defect observed in *defbl1* morphants. It was observed, by injecting *defbl1* morpholino into FliA-GFP transgenic fish), the *defbl1* morphants do not lose their macrophages (data not shown)(FliA acts as a marker for vasculature and macrophages (Lawson and Weinstein, 2002)). This function could be investigated in future studies.

4.5.2 *defbl1* and convergence extension movements

The work in this chapter implies that convergence and extension cell movements are disrupted in the *defbl1* morphants. This leads to a developmental delay which leaves the embryos with severe eye and brain phenotypes and a decreased anterior-posterior axis.

The phenotype observed in *defbl1* morphants is similar to that seen in the *knypek* mutant, which has been reported to mediate convergence extension cellular movements in the same pathway as *wnt11*(Topczewski et al., 2001). *kny* knockout embryos show a shortened anterior-posterior axis at 24hpf, along with broadened neural plate staining (as indicated by *dlx3* staining). However, these mutants do not appear to develop the same eye defects observed in *defbl1* morphants. The shared aspects of these mutant phenotypes suggest that *defbl1* may be involved in convergence extension control, however further investigation would be required to confirm this.

Recent work has suggested that the morpholino knockdown of cell essential genes often results in a non-specific phenotype similar to the *defbl1* morphants, characterised firstly by early brain necrosis (at 24hpf) followed by small eyes and a

developmental delay (Danilova *et al.*, 2010). As with the *defbl1* morphants, these cell essential knockdowns result in an up-regulation of *p53* which has been shown to mediate the non-specific response. While this may suggest that the observed *defbl1* phenotype is non-specific, it has been shown that where *p53* is mediating a non-specific response, the phenotype can be reversed in the presence of *p53* morpholino (Danilova *et al.*, 2010). Since the *defbl1* morphants retain their phenotype in the absence of functional p53 (data not shown), it can be deduced that although *p53* activation may contribute to the phenotype of the mutants, that it is not the causative element, nor is it necessary for the persistence of the phenotype.

4.5.3 Genome-wide analysis of *defbl1* morphants

The 12.5hpf timepoint was chosen to harvest morphant and control embryos for microarray analysis. This was chosen because the earliest time at which I could detect *defbl1* transcripts was at 12hpf and I hoped to utilise the array to capture a "snapshot" of the earliest expression changes to affect the morphant embryos. The presence of more than 6000 significantly differentially expressed genes in the morphants (with respect to control-injected embryos) implies that embryos could have been harvested at an earlier timepoint in order to omit some of the later downstream responses to the *defbl1* knockdown. In addition, the volume of genes affected suggests that although *defbl1* is undetectable by RT-PCR in embryos younger than 12hpf, that low, localised very low levels of expression might be occurring in younger embryos.

The microarray data represents a preliminary, broad attempt to identify any pathways that *defbl1* might be working in. Encouragingly, the data supported the developmental nature of the morphant defect and predictably showed an enhancement of expression changes in genes involved in neurological and optical development. Interestingly, the absence of large numbers of significantly affected infection and inflammation pathways also suggests that the observed morphant phenotype is not likely to be attributable to any decrease in immunity that *defbl1* might convey. This in turn, supports the findings in Chapter 3 that Defbl1 is not a potent antimicrobial peptide. This suggestion is also supported by the finding that

mildly bleaching embryos prior to morpholino injection has no effect on the morphant phenotype (data not shown), suggesting that microbial invasion is also not responsible for the morphant phenotype.

One drawback of the array is that it is impossible to determine whether the differential gene expression observed is a direct effect of the *defbl1* knockdown or merely one of many downstream effects. Although the keyword analysis (Table 4.3) suggests that the Wnt pathway is enriched amongst the changed genes, this still does not answer the question of whether *defbl1* works within this pathway or simply acts on it indirectly. Given the enrichment in changes of transcription factor expression (Table 4.2-3) and the high likelihood that *defbl1* is a secreted peptide, it may be that it affects a single (or multiple) cell surface receptor with multiple cellular targets. This would be concordant with the GO term analysis (Table 4.2) which identified transcription regulation as being highly enriched in morphants. The observation that *defbl1* appears to act in different ways at different developmental stages supports the idea that it binds more than one protein.

Finally, it should be noted that while the array provides interesting insights into the effects of *defbl1* knockdown, any concrete observations should be supported with an appropriate validation technique. In this study, a small cohort of affected genes were verified using qRT-PCR. However, since the qRT-PCR validation of one gene cannot validate a second unrelated gene in the array, extrapolating from any of the findings of this analysis would only be justifiable following validation of the specific genes involved.

4.5.4 *defbl1* and the Wnt-PCP pathway

As shown in Figure 4.15, a number of genes involved in the canonical and Wnt-PCP pathways were found to be differentially expressed, via microarray analysis, in *defbl1* morphants with respect to controls. Since the Wnt-PCP pathway has been implicated in the control of convergence extension (reviewed in Roszo *et al.*, 2009), it is intriguing that genes within this pathway were enriched in the microarray analysis, in particular with *wnt11* being confirmed (by qRT-PCR) as being down-

regulated in *defbl1* morphants. Table 4.4 lists all the Wnt-PCP pathway genes found to be differentially expressed in the *defbl1* morphants array (analysis carried out by Claire Smillie).

Gene name	Fold change in array
wnt11r	-1.91
rock2a	+1.065
vangl2	+1.0776
pp2a	+1.0037
pcdh8	+1.0065
dvl(2)	-1.0814
wnt5a	-1.0737
gpc4	1.2825
fzd7	1.0929
fzd2	-1.15
lpp	-1.3303
eaf1	-1.0853
eaf2	+1.0907
rhoa	+1.0164
drp2	-1.0336
prickle1	+1.0951
ntl	+1.052

Table 4.4 Genes involved in the Wnt-PCP pathway with altered expression in *defbl1* morphants (reproduced with permission from Claire Smillie) This table includes a selection of genes involved in the Wnt-PCP pathway whose expression was found to be altered in defbl1 morphants. The fold change in gene expression depicted in this table is low (<2 fold changes), however these fold changes were reported to be significantly different ($p<0.01$) to control-injected embryos.

As described previously, the *defbl1* morphant phenotype shares features of the *knypek* knockout mutant, however it also shares features of the *trilobite* (*strabismus*)

knockdown. Jessen *et al.*, 2002 reported a morpholino knockdown resulted in embryos with a convergence extension defect, resulting in a reduced anterior-posterior axis at 24hpf. While this is a similar phenotype to that observed in *defbl1* morphants, the eye defect is not replicated, indicating that while *defbl1* morphants may be involved in the Wnt-PCP pathway, that it may also influence other pathways.

An additional finding of relevance to the observed *defbl1* morphant phenotype is the report that *wnt7* transcripts (*wnt7aa*, *wnt7ab*, *wnt7ba*, *wnt7bb*) are expressed in the early zebrafish brain (Beretta *et al.*, 2011). The expression patterns reported in this study suggest a role for the wnt7 genes in neural specification and a role in early patterning is suggested as well. Since *wnt7ba* has been shown (and validated) to be down-regulated in *defbl1* morphants (Figure 4.16), it is possible that *defbl1* could function within the same pathway as *wnt7b* to effect both the observed developmental and neural phenotypes.

However, whether the identification of expression changes of genes present in the Wnt-PCP is a result of an earlier defect, or is itself the causal event in the mutant phenotype, is unknown.

4.6 CONCLUSIONS AND FUTURE DIRECTIONS

To conclude, in this chapter I have shown strong evidence supporting a novel role for zebrafish defensin *defbl1* in early development. Given the highly conserved nature of developmental genes at the gastrulation level, this work presents an interesting avenue for future work which could potentially be relevant to studies in other vertebrates and mammals.

Although there are many different approaches that could be taken to progress this work, I believe the most interesting data will yield from biochemical studies. The production of a Defbl1 antibody would facilitate highly informative pull down experiments to identify binding partners and any complexes that Defbl1 may form. Work of this nature would assist greatly in placing *defbl1* within a pathway. In addition, the presence of an antibody would allow for a detailed analysis of the expression pattern of *defbl1* to take place. While the study described in this chapter was informative, its main weakness is that the expression studies focussed on expression at the RNA level. Since RNA transcript changes do not always translate to a change in protein levels, antibody analysis on both whole embryos and embryonic and adult sections would be very useful, particularly in the earliest stages of *defbl1* expression. An antibody to Defbl1 is currently being produced in collaboration with another laboratory.

Further microarrays could also be carried out earlier in development, at the 11.5-12hpf timepoint to minimise the detection of downstream effects. This kind of analysis could be accompanied by whole cell quantitative proteomic assays to simultaneously detect the changes in protein levels associated with the mRNA transcript changes. Not only would the analyses serve to validate each other, but they would also provide interesting insight into global protein changes induced by *defbl1* knockdown.

The production of *defbl1* knock out fish would also provide insight into the function of the gene. This would comprehensively address the question of the effects of a complete knockdown in the morphant embryos and would also show whether the gene is essential for viability.

Finally, to gain further insight into the pathways within which *defbl1* functions, a chemical screen could be carried out on morphant embryos. The addition of small molecules from known kinase and phosphatase inhibitor libraries could be added to injected morphant embryos to observe whether topical application of the drug could reverse the earliest observed developmental phenotypes. Identifying a small molecule that could rescue the phenotype would place *defbl1* within a known pathway and could concentrate future studies into a relevant area.

Overall, this work provides a starting point for future studies into the molecular function of *defbl1*. It is unclear why or how a developmental function arose for a defensin-like gene, however the findings of further study may prove to be important in other vertebrate and mammalian defensin and development studies.

Chapter 5: Concluding Remarks

"The whole strenuous intellectual work of an industrious research worker would appear, after all, in vain and hopeless, if he were not occasionally through some striking facts to find that he had, at the end of all his criss-cross journeys, at last accomplished at least one step which was conclusively nearer the truth"

Max Planck

Antimicrobial resistance to current therapies is an increasing and dangerous problem. In my literature review in Chapter 1, I described how both opportunistic (for example *MRSA*) and pathogenic (for example, TB) microbes have acquired a worrying level of resistance to modern medicines. As illustrated by Swartz, 2004 in his depiction of the mortality rates of meningitis both before and after the introduction of antibiotics, effective antibiotic care is vital to maintaining basic standards of human health. The consequences, for example, of a multidrug resistant meningitis-causing *S.pneumoniae*, causing 100% mortality in those infected would be disastrous, but if bacteria keep evolving antibiotic resistance faster than drugs are produced, such situations could become a reality. The introduction of antibiotics revolutionised public health and their integrity must be preserved.

One potential source of novel antibiotics is the β-defensin family. This ancient set of antimicrobial peptides has adapted to fight various pathogens across evolution and a testament to their importance is their widespread conservation across organism as far-ranging as humans, platypus, zebrafish, fruit flies and even plants.

In Chapters 1 and 2 I outlined how the positive charge of the β-defensins, along with their hydrophobicity are thought to facilitate a mechanism whereby holes are punctured in microbial membranes. The nature of this proposed mechanism is such that microbes have to make significant changes to their cell membrane structure in order to gain resistance to the β-defensins, or alternatively some form of inhibitory mechanism would need to be acquired, thus reducing the likelihood of resistance emerging. In addition, since the β-defensins are bactericidal, few (if any) viable cells would be left behind following treatment, leaving little time for mutation or insensitivity to quickly occur.

Bearing these factors in mind, I began this study investigating murine Defb14, with a view to understanding more about the origins of its antimicrobial activity. Through my antimicrobial assays on the Defb14-1C^{V} deletion series, I showed that multiple N-terminal amino acid residues contribute to the antimicrobial activity of the molecule. In addition, my data indicated that a combination of dimerisation, charge and primary sequence are important for the maintenance of antimicrobial activity.

In order to further this study, I investigated how small peptide fragments could be modified in order to improve their antimicrobial activity. Since the Defb14 peptide fragment Defbl14-1C^{V}(6-17) was previously identified as being highly active (Reynolds *et al.*, 2010), I looked at the effects of cyclicisation and stereo isomerisation (by substituting L-amino acids for D-amino acids) on the peptide, with a view to improving its activity in the presence of serum. I found that while cyclicisation alone did not improve the antimicrobial activity, that stereoisomerism caused the peptide to perform significantly better under high serum conditions (Figure 2.14). This study suggests that a possible method of improving the antimicrobial activity of peptide antibiotics would be to investigate further the effects of D-form amino acids, and in particular, their resistance to serum proteases.

My work on the antimicrobial activity of Defb14 led to my interest in the zebrafish defensins, since at the beginning of this study, the genes were newly reported and had not been investigated beyond their initial reporting. I began investigating the zebrafish defensins with a primary aim to understand their role within the zebrafish immune system. However, at the same time I evaluated their suitability as antimicrobial agents and hoped they could tell us more about the nature of antimicrobial peptides.

In the work described in Chapter 3, using synthetic peptides, I identified the Defbl3 peptide as being a broad-spectrum antimicrobially active compound, whereas Defbl2 showed more specific low level activity solely against gram negative *P.aeruginosa*. Although I could detect the transcripts of all three zebrafish defensins in adult tissues (as was reported in Zou *et al.*, 2007 and Oehlers *et al.*, 2011), I also identified early expression of *defbl2* and *defbl3* from 6-7dpf, while *defbl1* was detected much earlier in 12hpf embryos.

The work presented in this thesis on antimicrobial activity presents a number of interesting directions in which new research could be carried out. With the Defb14 story, arguably the most exciting lead is the finding that Defb14-1C^{V}(7-17)CYC^{D}

peptide is highly antimicrobially active against *P.aeruginosa* in the presence of high levels of serum. This work could be quickly turned into an *in vivo* study whereby mice with infected wounds or systemic infections could be treated using the Defb14-$1C^{V}$(7-17)CYC^{D} as an antibiotic. If this peptide were to maintain its activity under physiological conditions, it could prove to be of great interest in the antibiotic development field.

My work on zebrafish antimicrobial activity also suggests that further work would be appropriate in this model to determine the role of the defensins within the zebrafish immune system. For example, it would be informative to investigate any role *defbl2* and *defbl3* have in the chemotaxis of adaptive immune cells (especially given that zebrafish express CCR6, a chemotaxis receptor in mammals, (Yang *et al.*, 1999)). Since the zebrafish is still being established as a relevant immunological model system, discovering more similarities between the zebrafish and mammalian immune systems would be beneficial and may yield interesting mammalian insights.

Finally, the antimicrobial inactivity of Defbl1 in combination with its early expression during development led to the proposal that *defbl1* has a role in early zebrafish development. The data presented in Chapter 4 indicated that *defbl1* directly or indirectly affects the convergence and extension cell movements beginning in gastrulation. A transient knockdown of *defbl1* was shown to result in a shortened anterior-posterior axis and brain and eye abnormalities which were rescuable upon co-injection of *defbl1* morpholino and mRNA.

This work is interesting because it identifies another unexpected role for the defensin superfamily. It also presents interesting evolutionary questions – for example, why and how did a defensin evolve a developmental role? In addition, is this developmental role confined to zebrafish and other teleosts, or is it conserved through to mammals? The reports of the involvement of murine *Defb19* in testis development (Menke and Page, 2002) and *Defb15* in *R.norvegicus* having a role in fertility and the survival of offspring (Zhao *et al.*, 2011) further suggest that a developmental role may exist for the defensins.

Elucidating a pathway for *defbl1* will be vital in understanding the observations reported in Chapter 4. Carrying out biochemical analysis on the peptide interactions, to uncover any binding partners and complexes formed would be informative, as would observing the peptide localisation within the embryo.

In conclusion, this thesis presents a wide-ranging study on the function of vertebrate defensins using both zebrafish and mouse defensins in order to gain further understanding of the larger defensin family. I have studied peptides from an applied point of view and have presented promising data on a highly antimicrobial and serum-resistant peptide fragment. I have also studied the β-defensins within the context of the whole organism and have demonstrated an important and varied endogenous role for the defensins discovered in zebrafish. The work in this thesis demonstrates the wide-ranging activity of the defensin superfamily and confirms their exciting applications to human medicine as well as their fascinating applications *in vivo*.

Chapter 6: Materials and Methods

nullius in verba

Nothing upon trust

Motto of the Royal Society

6.1 MOLECULAR METHODS

6.1.1 List of Solutions

Chemicals were obtained from Sigma-Aldrich unless otherwise stated. Dry chemicals and solutions were stored at room temperature unless otherwise stated.

Loading dye

20% Ficoll, 1% Orange G and 100mM EDTA

PCR, RT-PCR and qPCR primers

All primers were designed using Primer 3.0 software (Rozen and Skaletsky, 2000) and were purchased from Sigma-Aldrich. 10mM stocks were prepared by dissolving in TE buffer and 10μM working stocks were prepared by diluting in distilled water. Working stocks were stored at -20°C.

TBE buffer

Tris base	108g
Boric acid	55g
EDTA	9.3g

Buffer was made up to 500ml to make a 20X stock. 20X stock was diluted to a 1X working stock with distilled water.

TE buffer (obtained from the Human Genetics Unit Core Services)

10mM Tris.Hcl, 1mM EDTA

6.1.2 Polymerase chain reaction (PCR)

PCR reactions for sequencing and genotyping were performed using Recombinant Taq DNA polymerase from Invitrogen™. PCR for cloning was performed using high fidelity Phusion™ DNA polymerase (New England Biolabs).

PCR was carried out according to manufacturer's instructions with 1x reaction buffer, 4mM $MgCl_2$, 0.2μM forward and reverse primers10mM dNTPs, ~50ng DNA (1μl stock) and 1 unit Taq in a final volume of 25μl (made up with dH_2O).

High fidelity PCR was carried out in 1x HF buffer (New England Biolabs), 10mM dNTPs, 2μM $MgCl_2$, 1μM forward and reverse primers and 1 unit of DNA polymerase made up to 50μl with dH_2O.

PCR reactions were cycled according to a standard programme of 3 minutes denaturation at 94°C followed by 30 cycles of 45 seconds at 94°C, 30 seconds annealing at 58°C and 1 minute elongation at 72°C. A final elongation set was carried out for 10 minutes at 72°C.

High fidelity PCR reactions were cycled at 98°C, 30 seconds, and then on a two-step cycle (40x) 98°C for 10 seconds and 72°C for 45 seconds. A final elongation at 72°C was carried out for 10 minutes.

Sequences of primers used in this study are shown in Table 6.1.

6.1.3 RNA extraction

Tissue was homogenised in Trizol (Ambion) and transferred to screw-cap tubes. 100μl chloroform was added per ml Trizol, and tubes were shaken and incubated for 5 minutes. Samples were centrifuged at 13000 rpm, for 15 minutes and the upper, clear aqueous phase was transferred to a fresh tube. An equal volume of isopropanol was added and tubes were stored on ice for 15 minutes. RNA was precipitated by centrifugation at full speed for 15 minutes. The pellet was washed in 75% ethanol and resuspended in 30-50μl DEPC-treated dH_2O. RNA preparations were stored at -80°C. All incubations were carried out at 4°C.

Gene	Forward primer	Reverse primer	Source
ef1α	TTGAGAAGAAAATCGGTGGTGCTG	GGAACGGTGTGATTGAGGGGAATTC	Oehlers *et al.*, 2011
defbl1	AACATGAAGCCCCAGAGCAT	GAAAACTGGAGCTCCTGATC	Zou *et al.*, 2007
defbl2	ATATGAAGAAACTTGGCATG	AACCGCACAGCACAGCATCT	Zou *et al.*, 2007
defbl3	ATGAGCTACAATACGGGAAC	GTATCCGCATGTCCATCTCTG	Zou *et al.*, 2007
p53	CCCGGATGGAGATAACTTG	CACAGTTGTCCATTCAGCAC	Mei *et al.*, 2008
β-actin	TTTGAGCAGGAGATGGGAAC	GCAAGATTCCATACCCAGGA	Own design
bbc3	AACTCGGGCACGCCGAACAG	TCCTCAGCGGTCGAGCTGCT	Own design
fzd2	GTTGAGCGGAACTTTTCGAG	ACAGCACGGCAGTAACAGTG	Own design
gadd45a	GCATCCTGGCCACTGACGACG	CCCCCGAGGATGTTCGCGAG	Own design
prickle2	GCAGTAAAAGGCAGGAGCAC	CTGTGGAACTGCACAGAG GA	Own design
wnt7ba	CGGACGTCAAATATGGAGTG	GTTCATTAGCCTCCTTGCGT	Own design
wnt8a	GCTGGAAAATGGTCACGACT	AGTCGACCAGCTTCGTTGTT	Own design
wnt11	CCGTCTTCACCAATAGACCTTG	CCCAGTCTCTTCCCCTCAGT	Own design
eng2b	CCTGGAAACGTACTCCCTCA	TGGACAGCGGTTCTCTCTTT	Claire Smillie
fgf4	TAAAATCACCGGCGTACACA	AAGCTTCCCTTTGCTGTTCA	Claire Smillie
mdm2	CCTCCTCTTCCTCGACACTG	CGAAGGTTGTGTTGGGAGTT	Claire Smillie
wnt16	TCTCCGTTTACCCTTTGTGC	CTTCTCGGGCACTCCTACAG	Claire Smillie

Table 6.1 Primer sequences used for PCR, RT-PCR and qRTPCR

6.1.4 Reverse-transcription polymerase chain reaction (RT-PCR)

Freshly prepared or frozen RNA was used to prepare cDNA using the First Strand cDNA Synthesis (AMV) kit from Roche Applied Sciences, as per the manufacturer's instructions, using 1µg RNA. RT-PCR was then carried out as per the standard protocol for PCR.

6.1.5 Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was carried out using Brilliant II SYBR Green (Stratagene) mastermix according to the manufacturer's instructions. Samples were cycled in 20µl volumes according to the following general programme: 95°C for 10 minutes followed by 40 cycles of 95°C for 10 seconds, followed by 60°C for 45 seconds.

Cycling reactions were carried out in 384-well optical plates on an HT7900 lightcycler (Applied Biosystems). Data analysis to calculate relative fold change was carried out using $2^{-\Delta\Delta CT}$ conversions to convert CT values into the appropriate fold change. Calculations were carried out in Microsoft Excel.

6.1.6 Gel electrophoresis

PCR products were run on a 1.5% (w/v) (1.5g agarose in 100ml 1x TBE) agarose gel, supplemented with 1µl ethidium bromide and viewed over a UV illuminator. Samples were loaded with 1µl loading dye.

To extract bands for sequencing, gels were excised over a UV illuminator and samples were purified using a Qiagen gel extraction kit, following the manufacturer's instructions.

6.1.7 Sequencing

PCR reactions shown to have clear, single product bands were gel extracted (Section 6.1.6) and sent for sequencing by the MRC HGU Core Facility. Here, samples were

run on an ABI Prism 3730 Genetic Analyser using Big Dye v3.1 sequencing technology (kit obtained from Applied Biosystems). For consistency, sequencing samples were carried out in duplicate.

6.1.8 Cloning of *defbl1*

As described in Section 6.1.2, PCR for *defbl1* was carried out on whole embryo cDNA to amplify the transcript (primers shown in Table 6.1). PCR products and a pBluescript II SK+/- plasmid were digested using SacI and RsrII restriction enzymes (New England Biolabs) according to manufacturer's instructions. Digested fragments were ligated using T4 DNA ligase (Promega). A molar ration of 1:5 vector: insert DNA was incubated in 1x ligase buffer with 1 unit DNA ligase, made up to 10µl with dH_2O for 3 hours at room temperature. Ligated products were then transformed into competent cells (DH5α *E.coli*).

6.2 MICROBIOLOGICAL TECHNIQUES

All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

6.2.1 List of Solutions

Ampicillin (A9393, Sigma-Aldrich)

Foetal calf serum (FCS) (Sigma-Aldrich)

Isosensitest agar (Oxoid)

250ml stocks were made up by dissolving 7.85g agar powder in 250ml dH_2O and sterilising by autoclaving.

Isosensitest broth (Oxoid)

250ml stocks were made up by dissolving 5.85g powder in 250ml dH_2O and sterilising by autoclaving.

L agar (prepared by MRC HGU Core Services)

15g Difco agar

10g Bacto-tryptone

10g NaCl

5g yeast extract, made up to 1l with dH_2O

Luria broth: As supplied by MRC Human Genetics Unit Technical Services

10g Bacto-tryptone

10g NaCl

5g yeast extract, dissolved in 1l dH_2O

Phosphate buffer (10mM)

4.01ml 1M K_2HPO_4

0.99ml KH_2PO_4

0.5g glucose, dissolve in 500ml dH_2O

6.2.2 List of category 2 strains

Acinetobacter baumannii ATCC 19606

Candida albicans J2922

Edwardsiella tarda ATCC 15947

Escherichia coli ATCC 25922

Enterococcus faecalis ATCC 700802

Pseudomonas aeruginosa PAO1

Staphylococcus aureus ATCC 25923

Methicillin-resistant Staphylococcus aureus, MRSA J2918

6.2.3 Preparation, maintenance and growth of microbial stocks

Microbial stocks were stored at -80°c in 50% glycerol. For antimicrobial assays, microbes were streaked onto Isosensitest agar plates and grown overnight at 37°C. Plates were stored at 4°C for up to one week. Single colonies were inoculated into

10ml Isosensitest agar and incubated, with shaking at 225rpm at 37°C overnight. To ensure microbes were growing exponentially, 1ml overnight culture was inoculated into 10ml fresh Isosensitest broth and incubated for four hours prior to antimicrobial assays.

All strains were cultured in a level 2 biological containment suite.

6.2.4 *In vitro* antimicrobial assays

Approximately $1x10^6$ cells in exponential growth phase were incubated with various β-defensin derivatives in a 10mM phosphate buffer for 3 hours at 37°C (225rpm) (final volume, 100µl). Incubations were then spread onto Isosensitest agar plates and grown overnight. Plates were inspected for colonies the following morning and the percentage of organisms killed by the peptide was calculated. MBC values were taken as the lowest concentration that killed 99.99% initial inoculum.

All antimicrobial assays were performed at least in triplicate in independent experiments. For DTT treatments, peptides were incubated overnight in 10mM DTT to disrupt any dimers, prior to antimicrobial testing. The non-parametric Mann-Whitney U test was used to calculate statistical significance and data analysis was carried out using Microsoft Excel.

All peptides for antimicrobial assays were obtained from Derek Macmillan, UCL, where they were synthesised "in house".

6.2.5 Serum and salt sensitivity assays

To assess serum and salt sensitivity, antimicrobial assays were carried out using phosphate buffer supplemented with either 50mM, 100mM or 200mM NaCl or 1%, 5% or 10% foetal calf serum.

6.2.6 Transformation of competent cells

DH5α competent *E.coli* were obtained from Invitrogen and transformed according to the manufacturer's instructions. Briefly, 50µl cells were incubated with 100ng DNA on ice for 30 minutes. Cells were then heat-shocked at 42°C for two minutes and allowed to recover on ice for a further two minutes. Cells were then incubated in L broth for 1 hour and the entire culture was spun down and plated on selective (100µg/ml Ampicillin) L-agar plates overnight.

6.2.7 Isolation of plasmid DNA

Single colonies were picked off of selective agar plates and cultured for 12-16 hours overnight at 37°C, (5ml for minipreps, 50ml, maxipreps) in L broth supplemented with 50µg/ml L-ampicillin. Samples were shaken at 225rpm. Plasmid DNA was then extracted using Qiagen maxiprep or miniprep kits, according to the manufacturer's instructions. DNA was eluted in 30µl elution buffer.

6.3 ZEBRAFISH TECHNIQUES

6.3.1 List of Solutions

All solutions were obtained from Sigma-Aldrich unless otherwise stated.

E3 embryo medium (60x stock)

8.6g NaCl

2.45g $MgSO_4.7H_2O$

1.45g $CaCl_2.2dH_2O$

0.38g KCl, dissolved in 500ml dH_2O and stored at 4°C

Eosin

0.75% aqueous eosin

0.25% ethanol

0.05% acetic acid

Harris Haematoxylin

S212 , PolyScientific R&D

4% paraformaldehyde

2g paraformaldehyde dissolved in 50ml PBS, heated to 65°C until dissolved

6.3.2 List of Strains

$p53^{M214K/M214K}$ (dominant negative mutant) (described in Berghmans *et al.*, 2005)

AB wild type

6.3.3 Zebrafish maintenance and husbandry

Male-female pairs of fish were placed in mating tanks and were separated by a barrier overnight. The following morning, the barriers were removed and eggs were collected and washed in 1x E3 embryo medium. Fertilised eggs were washed in E3 embryo medium and incubated at 28°C.

To continue on fish lines, 5dpf (days post fertilisation) larvae were grown up in nursery tanks before being transferred to the adult system at 28dpf.

Embryos for sterile experiments were washed in a 0.07% bleach solution for five minutes before rinsing thoroughly in E3 embryo medium and repeating the process. At 24hpf, these embryos were dechorionated into autoclaved, sterile E3.

6.3.4 LPS and microbial treatment of zebrafish larvae

48hpf larvae were incubated in autoclaved E3 embryo medium containing 10ng/ml LPS for 6 hours at 28°C. Larvae were then sacrificed for RNA extraction.

For larvae treated with microbes, pre-incubations of embryos were cultured without cleaning at 28°C for 48 hours. This contaminated culture was then used to inoculate fresh 48hpf larvae for 6 hours at 28°C prior to sacrificing.

6.3.5 Morpholino and RNA injections

Embryos were collected (as above) and injected using glass capillary needles driven by nitrogen gas. Approximately 1nl DNA was injected directly into the cell (RNA) or yolk (morpholino) of single cell-stage embryos. Embryos at the two cell stage or greater were not injected. Injected embryos were incubated at 28°C and cleaned out regularly.

Morpholino oligonucleotides were designed by and obtained from Genetools. Morpholino sequences are shown in Table 6.2.

Morpholino	Sequence
T1	GGGCTTCATGTTGGATTCTTCAGAT
T2	TTCTTCAGATGATGGCAGTCCTGGG
S1	AGCAAACATACGAAACTTACAGTGC

Table 6.2 Morpholino sequences

6.3.6 *In vitro* transcription of mRNA for morpholino rescue

In vitro transcription was carried out using the *defbl1* cloned into the Bluescript II plasmid as described in Section 6.1.8. *In vitro* transcription was carried out using the mMessage mMachine® T7 Ultra kit (Ambion), according to manufacturer's instructions. This created capped transcripts with poly-A tails for additional stability.

RNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Nanodrop) and 5µl 40ng/µl aliquots were stored at -80˚C prior to use.

For the morpholino rescue experiment, 1µl stock RNA was mixed with 1µl 30µM T1 morpholino and injected directly into the embryonic cell. Control embryos injected with just morpholino were also injected into the cell (rather than the yolk) for consistency.

6.3.7 Imaging zebrafish embryos

Whole embryos were imaged using a Nikon stereo microscope and image data was captured and processed using IpLab spectrum software (Scanalytics corporation).

Embryonic sections were observed on a Zeiss Axioplan II microscope and data was processed on IpLab spectrum software.

Scripts to stamp scale bars onto images (written by Paul Perry, Human Genetics Unit) were used on this software.

6.3.8 Zebrafish embryo histology

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Embryos were dehydrated in methanol and then transferred to a series of three 100% ethanol washes. Embryos were then washed in xylene at room temperature, followed by xylene at 60°C prior to embedding in molten wax. Embryo blocks were allowed to harden overnight and were then 5µm sections were cut onto slides.

Slides were stained using haematoxylin and eosin. Embryos were first dewaxed in 3x5 min xylene washes and rehydrated in a series of decreasing alcohol baths (2x100%, 70%, 50%, 30% for 5 minutes each). Slides were washed thoroughly in running water and then stained in haematoxylin for 3 minutes. Excess dye was thoroughly rinsed off and slides were then differentiated in acid/alcohol, followed by lithium chloride. Eosin staining was then performed for 3 minutes. Slides were dehydrated in xylene and mounted using DePeX (BioChemika) and cover slips.

6.3.9 Zebrafish wholemount in situ hybridisation (WISH)

PCR primers were designed to amplify *myoD* with a T7 site placed at the 5' flank of the gene and T3 at the 3' to allow for both sense and antisense transcripts to be generated.

Probes for *hgg1* and *dlx3* were obtained from Susan Lynas, Human Genetics Unit and the *ntla* probe was obtained from Niki Wyatt, Human Genetics Unit.

In vitro transcription was carried out using T7 and T3 RNA polymerases (Promega) according to manufacturer's instructions, using Digoxygenin-11-UTPs (Roche Applied Science). Probes were then diluted 1:200 for use in wholemount in situ hybridisation. This procedure was carried out according to the widely used protocol described in Thisse *et al.*, 2008.

Anti-digoxygenin-AP Fab fragments (Roche Applied Science) was used as an antibody and following washing, embryos were stained in BM Purple (Roche Applied Science) for 1-2 hours.

Stained embryos were mounted in 70% glycerol and imaged under a stereomicroscope.

6.3.10 Zebrafish microarrays and analysis

Control and T1 morpholino-injected embryos were snap frozen at 12.5hpf for RNA extraction using Trizol, as described previously. Samples were then sent to Miltenyo Biotec for gene expression profiling using a one-colour Agilent Whole Zebrafish Genome Microarray.

Data obtained was arranged in a list of genes significantly up- or down-regulated in *defbl1* morphants with respect to controls. Data is based on the pooled microarray analyses obtained from two independent biological replicates.

The full list of genes differentially expressed in the array are included in the CD at the rear of this thesis.

The data sets obtained were analysed using DAVID software (http://david.abcc.ncifcrf.gov/) (Huang *et al.*, 2009a,b).

6.4 BIOINFORMATIC AND COMPUTATIONAL TECHNIQUES

6.4.1 List of programs and databases used

Blast

This was utilised with default settings, as described by Tatusova and Madden, 1999.

Clustal W (multiple sequence alignment)

Available at: http://www.ebi.ac.uk/Tools/msa/clustalw2/ (March 2011). As described by Higgins *et al.*, 1996.

6.4.2 Defensin gene prediction

Gene prediction was carried out using a UNIX interface. Multiple alignments of chicken, human and mouse β-defensins were performed using ClustalW and were then used to create a Hidden Markov Model using the HMMer program. A further program, Genewise was then used to systematically search the zebrafish genome (July 2007 version) for novel β-defensin-like genes. Hits were narrowed by their homology to known zebrafish ESTs to produce a shortlist of five genes. This work, including the running of Genewise was performed by Dr Colin Semple.

6.4.3 DAVID analysis (as described in Huang *et al.*, 2009a,b)

The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to analysis the data obtained in the microarray. Data was run through the "Gene ID Conversion" program to create a gene list which was run through a series of functional annotation tools. Genes were analysed according to the following annotation categories: Gene Ontology: Biological Function, Kegg_Pathways, PIR_Keywords and ZFIN_Anatomy.

Chapter 7: References

"Copying extensively from one source is plagiarism; copying extensively from several is research"

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Chapter 8: Appendices

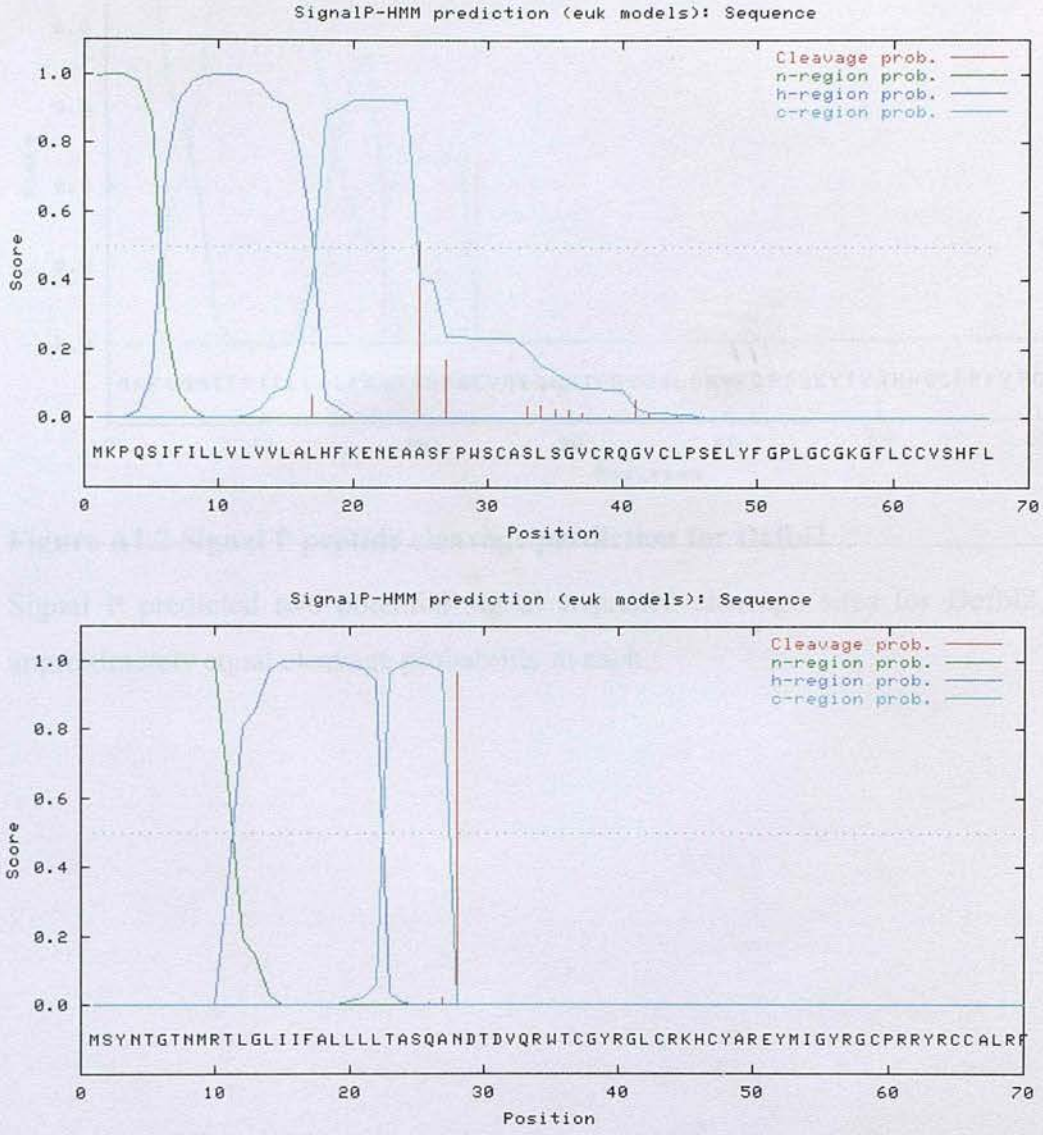

Figure A1.1 Signal P peptide predictions for Defbl1 (upper panel) and Defbl3 (lower panel)

Signal P predictions were run to confirm the observations of Zou *et al.*, 2007. Defbl1 and Defbl3 show clear predicted signal sequence cleavage sites, as depicted by the red "cleavage probability" line.

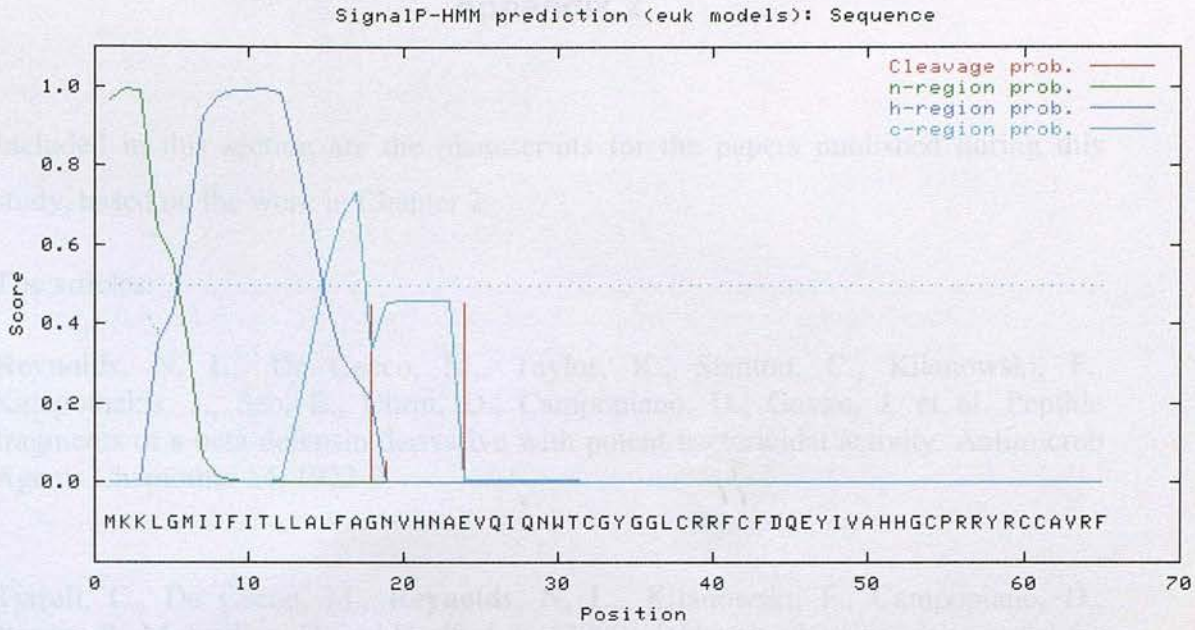

Figure A1.2 Signal P peptide cleavage prediction for Defbl2

Signal P predicted two potential signal sequence cleavage sites for Defbl2, with approximately equal cleavage probability at each.

Appendix 2

Included in this section are the manuscripts for the papers published during this study, based on the work in Chapter 2:

The articles:

Reynolds, N. L., De Cecco, M., Taylor, K., Stanton, C., Kilanowski, F., Kalapothakis, J., Seo, E., Uhrin, D., Campopiano, D., Govan, J. et al. Peptide fragments of a beta-defensin derivative with potent bactericidal activity. Antimicrob Agents Chemother 54, 1922-9.

Tyrrell, C., De Cecco, M., **Reynolds, N. L.**, Kilanowski, F., Campopiano, D., Barran, P., Macmillan, D. and Dorin, J. R. (2009). Isoleucine/leucine2 is essential for chemoattractant activity of beta-defensin Defb14 through chemokine receptor 6. Mol Immunol 47, 1378-82.

have been attached. Appropriate consent was obtained for the reprinting of these journal articles.

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Peptide Fragments of a β-Defensin Derivative with Potent Bactericidal Activity▽

Natalie L. Reynolds,[1]† Martin De Cecco,[2]† Karen Taylor,[1] Chloe Stanton,[1] Fiona Kilanowski,[1] Jason Kalapothakis,[2] Emily Seo,[2] Dusan Uhrin,[2] Dominic Campopiano,[2] John Govan,[3] Derek Macmillan,[4] Perdita Barran,[2] and Julia R. Dorin[1]*

MRC Human Genetics Unit, IGMM, Edinburgh EH4 2XU, United Kingdom[1]; School of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, United Kingdom[2]; Cystic Fibrosis Laboratory, Medical Microbiology, University of Edinburgh, Edinburgh EH16 4SB, United Kingdom[3]; and Department of Chemistry, Christopher Ingold Laboratories, University College London, London WC1H 0AJ, United Kingdom[4]

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β-Defensins are known to be both antimicrobial and able to chemoattract various immune cells. Although the sequences of paralogous genes are not highly conserved, the core defensin structure is retained. Defb14-1C^{V} has bactericidal activity similar to that of its parent peptide (murine β-defensin Defb14) despite all but one of the canonical six cysteines being replaced with alanines. The 23-amino-acid N-terminal half of Defb14-1C^{V} is a potent antimicrobial while the C-terminal half is not. Here, we use a library of peptide derivatives to demonstrate that the antimicrobial activity can be localized to a particular region. Overlapping fragments of the N-terminal region were tested for their ability to kill Gram-positive and Gram-negative bacteria. We demonstrate that the most N-terminal fragments (amino acids 1 to 10 and 6 to 17) are potent antimicrobials against Gram-negative bacteria whereas fragments based on sequence more C terminal than amino acid 13 have very poor activity against both Gram-positive and -negative types. We further test a series of N-terminal deletion peptides in both their monomeric and dimeric forms. We find that bactericidal activity is lost against both Gram types as the deletion region increases, with the point at which this occurs varying between bacterial strains. The dimeric form of the peptides is more resistant to the peptide deletions, but this is not due just to increased charge. Our results indicate that the primary sequence, together with structure, is essential in the bactericidal action of this β-defensin derivative peptide and importantly identifies a short fragment from the peptide that is a potent bactericide.

β-Defensins are believed to be important components in innate immunity. They are expressed predominantly at mucosal surfaces and the reproductive tract. They are a multigene family which has ~40 members spread over five genomic loci in humans (18). They possess a strong, broad-spectrum antimicrobial action *in vitro* and also have been shown to be chemoattractants for various immune cells (23, 24). Some β-defensins are rapidly induced by exposure to lipopolysaccharide or Th1 cytokines, while human β-defensin 1 (hBD1) expression is mostly constitutive (14). Recently, increased copy number of the β-defensins at the chromosome 8 locus has been associated with an increased risk of psoriasis (7). Conversely, decreased copy number of this cluster has been associated with an increased risk of inflammatory bowel disease (5).

Defensins have a canonical six-cysteine motif, but the remaining sequence of these short peptides is not highly conserved. The consensus is $X_{2\text{-}10}CX_{5\text{-}7}(G/A)XCX_{3\text{-}4}CX_{9\text{-}13}CX_{4\text{-}7}CCX_n$, where X is any residue, G/A is glycine or alanine, and C is cysteine. The spacing and connectivity of the six cysteines separate α-defensins from β-defensins, and although highly conserved throughout evolution, the canonical six-cysteine motifs in either defensin class are not required for antimicrobial activity (2, 11, 20, 23). The canonical disulfide bridges may be important for protection of the peptide *in vivo*, as has been demonstrated for α-defensins (11).

Evolutionary studies of the mouse and human genomes have revealed that *Defb14* is the mouse orthologue of human β-defensin 3 (*DEFB103*) (19). The peptide encoded by *Defb14* has salt-resistant, wide-range antimicrobial activity very similar to that of the peptide encoded by *DEFB103* (human β-defensin 3-hBD3) (6, 16, 20). Like all defensins, hBD3 has an antiparallel β-sheet scaffold with a short N-terminal α-helix (17), and this structure is conserved throughout evolution.

Given the promiscuity of the endogenous peptides, understanding the structure-activity relationships of β-defensins is essential. Work in this area aims to find key information for designing novel synthetic antibiotics and for clarifying their function in immunity. There is some controversy as to the mode of action of defensins with membrane disruption by some mechanism seeming to occur. However, studies with hBD3 in *Staphylococcus aureus* have revealed the occurrence of rapid killing and at the same time blockage of all biosynthetic pathways, although significant depolarization of the bacterial membranes was not observed and permeabilization was incomplete (1).

Wu et al. (23) elegantly used directional disulfide bonding to demonstrate that the disulfide connectivities influenced the chemoattractant potency of hBD3 but not the antibacterial effect. We have also shown that hBD3 or Defb14 peptides with

* Corresponding author. Mailing address: MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, United Kingdom. Phone: (44) 131 467 8411. Fax: (44) 131 467 8456. E-mail: julia.dorin@hgu.mrc.ac.uk.

† N.L.R. and M.D.C. contributed equally to the study.

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TABLE 1. Sequences of Defb14-derived peptides

Peptide	Sequence[a]
Defb14	FLPKTLRKFF**C**RIRGGR**C**AVLN**C**LGKEEQIGR**C**SNSGRK**CC**RKKK
Defb14-1C^{V}	FLPKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1)	LPKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-2)	PKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-5)	LRKFFARIRGGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-8)	FFARIRGGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-11)	RIRGGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-14)	GGRAAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-17)	AAVLNALGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}Δ(1-23)	LGKEEQIGRASNSGRK**C**ARKKK
Defb14-1C^{V}(1-23)	FLPKTLRKFFARIRGGRAAVLNA
Defb14-1C^{V}(1-10)	FLPKTLRKFF
Defb14-1C^{V}(6-17)	LRKFFARIRGGR
Defb14-1C^{V}(18-23)	AAVLNA
Defb14-1C^{V}(14-23)	RGGRAAVLNA
Defb14-1C^{V}(12-23)	RIRGGRAAVLNA
Defb14-1C^{V}(13-34)	IRGGRAAVLNALGKEEQIGRAS

[a] The sequences are given using single-letter abbreviations for amino acid residues; cysteine residues are in boldface. Deletion peptides are based on the Defb14-1C^{V} active peptide used previously (20).

all but one cysteine being replaced by alanine were still active as both a chemoattractant and an antimicrobial. The remaining cysteine was at position V of the canonical six-cysteine motif (-C^{I}-C^{II}-C^{III}-C^{IV}-C^{V}-C^{VI}-), where the cysteines are interspersed with a variable number of amino acid residues. Hoover et al. (8) demonstrated that peptides from the C-terminal region of hBD3 had potent activity against *Escherichia coli* and *Pseudomonas aeruginosa* but not *S. aureus*. The shortest peptide was a decapeptide based on sequence from the C-terminal end of the molecule (RGRKSSRRKK). The two adjacent cysteines in the native peptide were replaced with serines to eliminate the potential to form disulfide bonds (8). Further work using modifications of this decapeptide sequence has shown that its antimicrobial activity is more complex than just being a function of charge (13). In addition, work on an hBD3 derivative where the cysteines of the full-length mature peptide are changed to alanines has revealed that this peptide is as active as the parent peptide against Gram-positive and -negative bacteria but is now sensitive to the ionic strength of the medium (3). As the cysteine content of the peptide does not affect the antimicrobial activity of the peptide, we have focused on the Defb14-1C^{V} analogue (20). This peptide has all the cysteines replaced with alanines except Cys^{40}, which resides at position V of the six-cysteine motif. In addition, it can easily be controlled to form monomer or dimer species. We have previously shown that this peptide analogue has the same salt-insensitive bactericidal activity as the parent Defb14 peptide (20). In addition, we divided the active Defb14-1C^{V} peptide into an N-terminal and a C-terminal fragment and found that the C-terminal half (residues 24 to 45) was a very poor antimicrobial compared to the N-terminal fragment (residues 1 to 23), which was potent. In order to further dissect the residues important for the antimicrobial activity of Defb14-1C^{V}, we created a library of peptide fragments. We show that the antimicrobial activity of the most N-terminal residues is most potent, and deletion of these residues reveals that the points at which the activity is most significantly lost vary for different bacterial strains. We conclude that both the sequence and structure that a given sequence imposes are important for antimicrobial activity.

MATERIALS AND METHODS

Peptide synthesis and purification. All peptides were chemically synthesized by standard solid-phase methodology. Defb14 and Defb14-1C^{V} were obtained from Chemical Synthesis Services-Albachem Ltd. (Gladsmuir, United Kingdom). Disulfide connectivities were determined by proteolysis and peptide mass mapping by following the procedures outlined by Campopiano et al. (2). The sequences are shown in Table 1. The defensin 14-inspired peptides, including additional synthesis of Defb14-1C^{V}, were made in-house using automated peptide synthesis. This was carried out on an Applied Biosystems model 433A peptide synthesizer using Rink amide-AM resin for peptide amides, preloaded NovaSyn TGT resin for peptide acids, and 9-fluorenylmethoxy carbonyl (Fmoc) amino acids, all from Novabiochem. All truncated Defb14-1C^{V} peptides were synthesized with an acetyl group at the N terminus, rather than the free amino group, to best represent the conformation of the terminal amino acid. Liquid chromatography (LC) mass spectra confirming identity and purity were obtained on a Micromass Quattro LC mass spectrometer. Semipreparative high-performance liquid chromatography (HPLC) was performed using a Phenomenex Luna C_{18} column and a gradient of 5 to 95% acetonitrile (containing 0.1% trifluoroacetic acid) over 45 min (flow rate of 3.0 ml/min). All other chemical reagents were obtained from Aldrich. Automated solid-phase peptide synthesis was carried out on a 0.05-mmol scale using 0.5 mmol of each Fmoc amino acid per coupling reaction and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole as coupling reagents. Coupling time was 0.5 h. Peptide products were cleaved from the resin with 95% trifluoroacetic acid, 2.5% ethanedithiol, and 2.5% water for 3 h, the resin was filtered off and washed with trifluoroacetic acid, and filtrate was poured into diethyl ether (10 volumes). Following centrifugation (3,000 rpm at 15 min), the precipitate was resuspended in ether (5 volumes) and recentrifuged (3,000 rpm at 15 min). The crude peptides were dissolved in water and loaded directly onto a semipreparative HPLC column. Peptide fractions were identified by mass spectrometry (MS) and lyophilized.

Bactericidal assays. Bactericidal assays were carried out as previously described (20). Briefly, test organisms were grown to mid-logarithmic phase in Iso-Sensitest broth (Oxoid) growth medium and then diluted to between 1×10^{6} CFU/ml and 5×10^{6} CFU/ml in 10 mM potassium phosphate containing 1% (vol/vol) Iso-Sensitest broth, pH 7.4. Different concentrations of test peptide were incubated in 100 μl of cells (1×10^{5} to 5×10^{5} CFU) at 37°C for 3 h. Reduction of the peptides, where performed, was done by adding 10 mM dithiothreitol (DTT) and incubating the peptides at room temperature overnight. The oxidation state of each peptide was determined by mass spectrometry. Tenfold serial dilutions of the incubation mixture were spread on Iso-Sensitest plates and incubated at 37°C, and the numbers of CFU were determined the

TABLE 2. MBCs of Defb14-1C^V derivatives against a range of Gram-negative and Gram-positive bacteria

Organism and strain	MBC (μM) for peptide[a]: Defb14	Defb14-1C^V	Defb14-1C^V (1-23)	Defb14-1C^{V}Δ (1-23)	Defb14-1C^V (1-10)	Defb14-1C^V (6-17)	Defb14-1C^V (14-23)	Defb14-1C^V (13-34)
Gram negative								
P. aeruginosa PAO1	**0.3**	**0.3**	**0.6**	>20.5	**1.2**	**1**	>48	>22
A. baumannii ATCC 19606	**0.6**	**0.6**	**1.2**	>20.5	**19**	**1**	>48	>22
Burkholderia cenocepacia J2315	>20	>20	>20	>20.5	>40	>33	>48	>22
E. coli ATCC 25922	**0.6**	**0.6**	**1.2**	>20.5	**9.6**	**2**	>48	>22
Gram positive								
E. faecalis ATCC 700802	**1.2**	**1.2**	**2.4**	>20.5	**19**	**16.5**	>48	>22
S. aureus ATCC 25923	**0.6**	**0.6**	**1.2**	>20.5	38.6	**9.6**	>48	>22
MRSA J2918	**1.2**	**1.2**	**2.4**	>20.5	38.6	**4.8**	>48	>22

[a] Bactericidal activity is located in the N-terminal sequence of the β-defensin peptide. The region of the molecule that the peptide sequence is derived from is indicated beneath its name by gray shading. Boldface indicates MBC of less than 20 μM.

following day. The minimum bactericidal concentration (MBC) is the concentration of peptide at which we observed >99.99% killing of the initial inoculum. All assays were performed in duplicate and repeated on three independent occasions. The minimum bactericidal concentration was obtained by taking the mean of all of the results, and experimental errors were within one doubling dilution. Significant differences between MBCs were determined using a Mann-Whitney U test.

Molecular hydrophobicity analysis. The relative molecular hydrophobicity of each monomeric peptide was evaluated by reverse-phase HPLC-MS using an Ultimate 3000 LC system equipped with a Famos autosampler (Dionex) and a VG Platform II mass spectrometer. A total of 30 μl of each sample, containing 50 μM peptide in 10 mM dithiothreitol (DTT) and 50 μM melittin (Sigma) as an internal standard, was injected onto a Waters Symmetry C_{18} column (3.9 by 150 mm). DTT was used to prevent dimerization of any of the peptides via cysteine oxidation; this was confirmed by mass analysis following the LC separation. Initial conditions were as follows: 100% eluent A (96% water, 3% acetonitrile, 1% formic acid); 0% eluent B (4% water, 95% acetonitrile, 1% formic acid); and flow rate, 1 ml min^{-1}. Samples were eluted from the column by using a linear gradient of 0 to 40% eluent B over 40 min. The flow was split 1/50 prior to introduction to the electrospray ionization source of the mass spectrometer. In order to assess the relative molecular hydrophobicities, we compared the retention time of each peptide to that of melittin.

ΔG, the hydrophobicity score (kcal/mol) of monomeric peptides in water, was calculated using the scale of Wimley and White (22), where greater hydrophobicity is indicated by a less negative value.

Helical wheel projections. Helical wheel projections were constructed with the aid of the program at http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.

RESULTS

Bactericidal activity of peptide fragments. In order to narrow the antibacterial activity of Defb14-1C^V to a particular region of the molecule, we synthesized three overlapping fragments (1 to 10 [1-10], 6 to 17 [6-17], and 14 to 23 [14-23]) of the antimicrobially active N-terminal Defb14-1C^V. These fragments (see Table 1 for sequences) were tested against a panel of Gram-positive and Gram-negative bacteria. We determined the minimum bactericidal concentration (MBC) values for these peptide fragments (Table 2), i.e., the minimum concentration at which less than 99.99% of the initial inoculum is viable.

The 23-amino-acid N-terminal fragment Defb14-1C^V(1-23) has bactericidal activity very similar to that of Defb14-1C^V (as previously reported under the name D14ip 1 [20]) against *Pseudomonas aeruginosa* PAO1 and *S. aureus* ATCC 25923. The peptide fragment with the first 10 amino acids of Defb14-1C^V [Defb14-1C^V(1-10)] shows good activity against *P. aeruginosa* PAO1 (MBC of 1.0 μM), *Acinetobacter baumannii* (MBC of 0.58 μM), and *E. coli* (MBC of 2.3 μM), and these are within one doubling dilution of the Defb14-1C^V(1-23) fragment and comparable to the parental peptide activity against these strains. The Defb14-1C^V peptide fragment with the 12 internal amino acids [Defb14-1C^V(6-17)] also has a strong bactericidal activity against the three Gram-negative strains tested. The peptide fragments containing amino acids 14 to 23 and 13 to 34 do not achieve an MBC against the bacteria at the concentrations tested here. Interestingly, only one of the two most N-terminal peptides has good activity against the Gram-positive strains tested. Only the fragment with residues 6 to 17 has the ability to kill all the Gram-positive strains at a concentration less than 19 μM (~30 μg/ml). Defb14-1C^V(6-17) had good activity against methicillin-resistant *S. aureus* (MRSA) strain J2918 (MBC of 4.8 μM), comparable with that of the Defb14-1C^V(1-23) fragment, and its MBC against *S. aureus* was 8.2 μM. We previously reported that Defb14-1C^V and the active N-terminal fragment Defb14-1C^V(1-23) had salt-insensitive activity against *P. aeruginosa* (20). Interestingly, the bactericidal activities of both Defb14-1C^V(1-10) and Defb14-1C^V(6-17) against *P. aeruginosa* are unchanged with increasing salt concentrations of up to 200 mM NaCl (data not shown).

Bactericidal activity of N-terminal deletions. In order to further dissect the antimicrobial sequence within the N-terminal region of Defb14-1C^V and in the context of the molecule with the C-terminal region included, we made a series of N-terminal deletions (see Table 1 for sequences). As the deletions are based on Defb14-1C^V, they all have a single cysteine residue (Cys^{V40}). This enabled us to examine both monomeric and covalent dimeric forms, with the latter occurring via a disulfide bridge. Following oxidation, the peptides spontaneously form a homodimer through the cysteine-cysteine disulfide bridge and reduction with DTT leaves the peptide in the reduced form, as verified by mass spectrometry, and this state is maintained throughout the assay (data not shown). We determined the MBCs of both the dimeric and monomeric molecules against three Gram-negative and three Gram-positive bacterial strains for all the deletion peptides (Table 3).

As the deletions progress, the MBC increases in all cases,

indicating loss of bactericidal activity. The points at which the bactericidal activity significantly reduces are not the same for all the bacterial strains. Deletion of 14 amino acids results in the monomeric form of the peptide being unable to kill any of the six bacterial strains at the concentrations tested. The critical point for activity against *P. aeruginosa* and *A. baumannii* ATCC 19606 is between Defb14-1C^{V}Δ(1-11) and Defb14-1C^{V}Δ(1-14). However, against another Gram-negative strain (*E. coli*), deletion of 8 amino acids results in the bactericidal activity falling 4-fold to 10 μM (37.3 μg/ml). Against Gram-positive *S. aureus* ATCC 25923 and MRSA J2918, the bactericidal activities drop significantly after deletion of 11 amino acids. However, deletion of only 5 amino acids results in the monomeric peptide significantly losing activity against *Enterococcus faecalis*.

The dimeric peptides are more robust than the monomers in their ability to kill the bacteria when the N-terminal amino acids are progressively deleted. All four strains tested show a significant increase in the MBC of the monomeric species before the dimeric species' MBC is affected. *P. aeruginosa* is killed at only 1.4 μM with the Defb14-1C^{V}Δ(1-14) dimeric species, whereas the monomeric form is not able to kill the bacterium at 16.5 μM. The same effect was observed with *E. coli* and *A. baumannii*, where the monomer species loses bactericidal activity before the dimer forms. The Gram-positive strains also display this effect. For Defb14-1C^{V}Δ(1-11), there is a 6-fold increase in the amount of monomer required to kill *S. aureus* ATCC 25923 compared to the amount of dimer (5.9 μM versus 0.9 μM), and with the same peptide, the monomer MBC against MRSA is >10 μM, compared to 1.5 μM for the dimer MBC.

The dimer deletion peptide activities against *P. aeruginosa* in increasing sodium chloride concentrations of up to 200 mM were tested. Like the parent peptide and peptide fragments 1 to 10 and 6 to 17, the bactericidal activities of deletion peptides up to and including Defb14-1C^{V}Δ(1-5) were not significantly affected by addition of sodium chloride at concentrations of up to 200 mM. However, deletion peptide Defb14-1C^{V}Δ(1-8) had a significantly ($P < 0.01$) raised MBC of 10 (±0.0) μM in 10 mM sodium chloride compared to 1.7 (±0.0) μM in 0 mM NaCl. The subsequent deletions also showed significantly ($P < 0.01$) increased MBCs of >10 mM in 100 mM NaCl, indicating that loss of the N-terminal region of Defb14-1C^{V} rendered these peptides salt sensitive.

In order to check whether degradation of the peptides by bacterium-specific proteases could explain the loss of bactericidal activity, the integrity of the inactive peptide Defb14-1C^{V}Δ(1-14) was evaluated by LC-MS following incubation with *S. aureus* ATCC 25923. After 4 h, the majority of the peptide remained intact (peak area of 66% relative to that of sample prior to incubation; data not shown), indicating that proteolysis of the peptide by the bacterial strain does not explain its inactivity.

Charge versus bactericidal activity. In an effort to determine whether charge rather than the primary sequence of the peptides had an effect on the ability to kill bacteria, we considered the net charge of each sequence in the peptide library we had synthesized. Figure 1 shows the charge of monomer molecules (and dimer molecules where relevant) and is annotated according to which peptides are active against *P. aeruginosa*

TABLE 3. MBCs for monomer and dimer forms of the N-terminal deletion peptides

Peptide	MBC (μM)[a]											
	Pseudomonas aeruginosa PAO1		*Escherichia coli* ATCC 25922		*Acinetobacter baumannii* ATCC 19606		*Staphylococcus aureus* ATCC 25923		MRSA J2918		Enterococcus faecalis ATCC 700802	
	Monomer	Dimer	Monomer	Dimer	Monomer	Dimer	Monomer	Dimer	Monomer	Dimer	Dimer	Monomer
Defb14-1C^{V}	0.3 (±0.0)	0.2 (±0.0)	0.6 (±0.0)	0.3 (±0.0)	0.6 (±0.0)	0.3 (±0.0)	1.4 (±0.5)	0.5 (±0.0)	1.2 (±0.0)	0.6 (±0.0)	1.2 (±0.2)	0.6 (±0.2)
Defb14-1C^{V}Δ(1)	ND	0.3 (±0.0)	ND	1.0 (±0.6)	ND	0.6 (±0.0)	ND	0.7 (±0.0)	ND	0.7 (±0.0)	ND	0.8 (±0.2)
Defb14-1C^{V}Δ(1-2)	0.7 (±0.0)	0.5 (±0.2)	3.3 (±0.8)	0.6 (±0.2)	1.7 (±0.4)	0.6 (±0.0)	1.0 (±0.3)	0.8 (±0.2)	2.1 (±0.4)	0.8 (±0.6)	4.2 (±0.8)	1.0 (±0.2)
Defb14-1C^{V}Δ(1-5)	0.7 (±0.0)	0.6 (±0.1)	2.5 (±0.0)	1.1 (±0.4)	ND	1.0 (±0.2)	1.4 (±0.0)	1.1 (±0.4)	2.5 (±0.0)	1.7 (±0.8)	ND	1.25 (±0.0)
Defb14-1C^{V}Δ(1-8)	1.1 (±0.4)	0.7 (±0.4)	**10 (±0.0)**	2.5 (±0.0)	2.9 (±1.1)	1.3 (±0.6)	1.5 (±0.0)	1.1 (±0.3)	2.2 (±0.4)	1.3 (±0.0)	**>5**	1.9 (±0.6)
Defb14-1C^{V}Δ(1-11)	1.7 (±0.0)	0.7 (±0.2)	>10	>5	2.5 (±0.0)	1.0 (±0.2)	**5.9 (±0.5)**	0.9 (±0.3)	**>10**	1.5 (±1.1)	>5	>5
Defb14-1C^{V}Δ(1-14)	**>15.2**	1.4 (±0.3)	>10	>5	**>10**	2.5 (±0.0)	>15.2	>7.6	>10	6.7 (±3.3)	>10	>5
Defb14-1C^{V}Δ(1-17)	>16.5	2.6 (±1.0)	>10	>5	>10	3.3 (±0.8)	>16.5	>8.3	>10	>5	>10	>5
Defb14-1C^{V}Δ(1-23)	ND	>10	ND	>10	ND	>10	ND	>10	ND	>10	ND	>10
Defb14-1C^{V}(18-23)	>83.5	NA	ND	ND	ND	ND	>83.5	NA	ND	NA	ND	ND

[a] Loss of bactericidal activity upon peptide N-terminal deletion is bacterial strain dependent. Sequences of peptides are given in Table 1. Peptides are in either the monomer (DTT treated) or dimer form. Values in parentheses are standard errors of the means. Values in boldface indicate that the MBC for the monomeric form is significantly different ($P < 0.01$) from that of the Defb14-1C^{V} monomer. ND, not done; NA, not applicable.

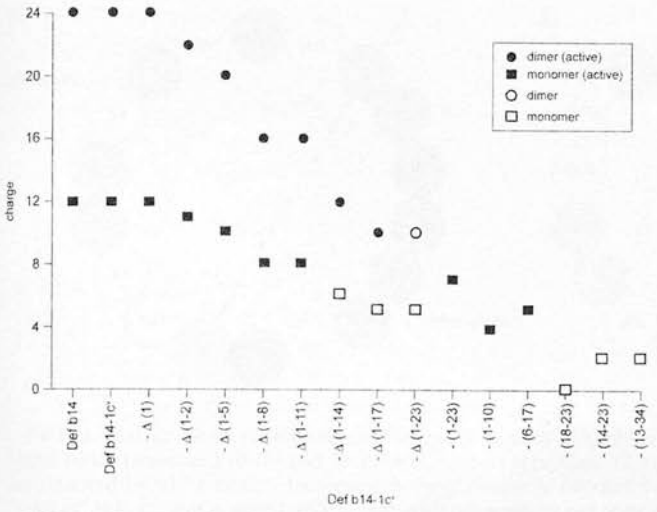

FIG. 1. Charge and structure are important for antimicrobial activity of β-defensin derivatives. The Defb14-1C^{V} derivative library was plotted according to the net charge (protonation state) of the synthesized peptides at a neutral pH. This takes into account the number of basic versus acidic amino acids and also the termini. The key indicates whether peptides are active bactericides and dimers or monomers.

PAO1 with an MBC of less than 5.0 μM (shown as filled dots or squares in the figure). All the inactive peptides (shown with open dots or squares) possess a charge of less than +11; however, six active peptides also have a charge of less than +11, with Defb14-1C^{V}(1-10) having a charge of only +5 and an MBC of 1.2 μM. The Defb14-1C^{V}Δ(1-17) dimer has a charge of +10 and is a potent antimicrobial agent with a low MBC, but in contrast, the Defb14-1C^{V}Δ(1-23) dimer has an equal charge but is an inactive antimicrobial with an MBC in excess of 10 μM.

Hydrophobicity versus bactericidal activity. We also assessed the hydrophobicity of the deletion peptides both according to their primary sequences via the Wimley-White scale for proteins at a membrane interface (Fig. 2a) (22) and experimentally by measuring their retention times by reverse-phase HPLC (Fig. 2b). This second method is sensitive to the conformation of the free peptide in solution. However, we observe little difference between the hydrophobicities of these peptides, as gauged by either method (Fig. 2). The hydrophobicity via HPLC does decrease as the length of the peptide decreases, with the exception of Defb14-1C^{V}Δ(1-17), which shows a slight increase relative to that of Defb14-1C^{V}Δ(1-14). The observed differences in bactericidal activity with these peptides cannot be attributed to any dramatic difference in the hydrophobicity of the free peptide.

Structural change as determined by helical wheel projection. Figure 3 shows a helical wheel projection of Defb14-1C^{V} (residues 1 to 18) which predicts that the N-terminal region of the full-length peptide has the potential to form an α-helix with charged residues concentrated on one face of the helix and hydrophobic residues on the other. The distinction between hydrophilic and hydrophobic faces is less clear in helical wheel projections of residues 12 to 29 and 15 to 32, which correspond to the N-terminal regions of the Defb14-1C^{V}Δ(1-11) and Defb14-1C^{V}Δ(1-14) peptides, respectively. This suggests a

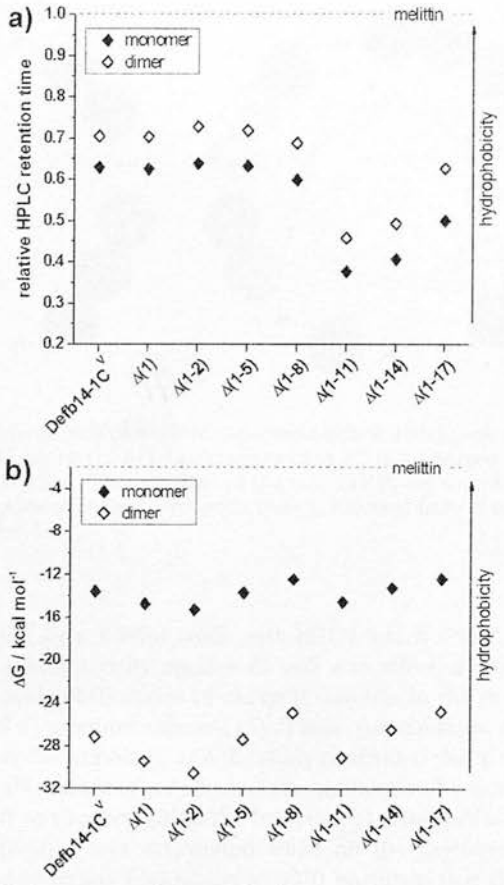

FIG. 2. Assessment of the relative hydrophobicities of the Defb14-1C^{V} N-terminal deletion series. (a) The retention times of each N-terminal deletion peptide, relative to that of melittin, are measured for both the monomer and dimer under reducing and nonreducing conditions, respectively. Increased relative retention time is indicative of more hydrophobic character, since under a reversed-phase gradient hydrophobic peptides will be retained longer on the C_{18} column. For comparison, panel b shows the theoretical hydrophobicity score (in kcal mol^{-1}) of each peptide in water, calculated using the scale of Wimley and White (22). Greater hydrophobicity is indicated by a less negative value.

lesser propensity to form an α-helix, which we have also shown by circular dichroism (CD) (21), and may account, in part, for the diminished activity of the truncated peptides. There is little difference in these projections for residues 12 to 29 and 15 to 32.

DISCUSSION

In order to further dissect the basis of the potent bactericidal activity of the N-terminal region of the Defb14-1C^{V} derivative, we synthesized several overlapping fragments of the 23-amino-acid region and a series of Defb14-1C^{V}-derived peptides with progressive N-terminal deletions. We determined their MBCs against a panel of Gram-positive and -negative bacterial strains. Although the Defb14-1C^{V}(6-17) fragment is a strong bactericide, the deletion series reveals that there does not appear to be one short sequence responsible for activity against all strains.

Primary sequence determines bactericidal activity against different bacteria. We show that the more C-terminal fragment

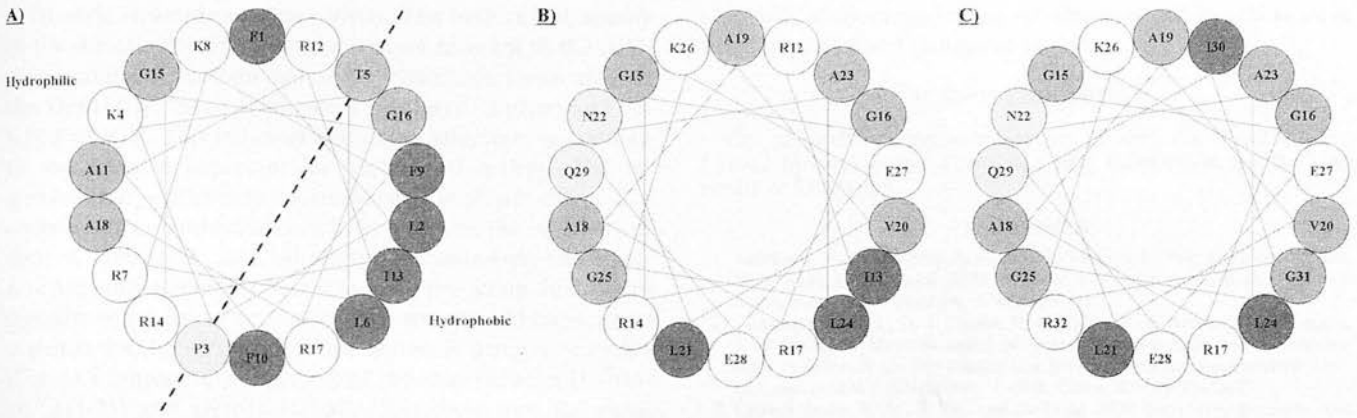

FIG. 3. Helical wheel projections of N-terminal regions of Defb14-1C^V and inactive/active derivatives. Schematic helical wheel projections of Defb14-1C^V (residues 1 to 18) (A), Defb14-1C^{V}Δ(1-11) (residues 12 to 29) (B), and Defb14-1C^{V}Δ(1-14) (residues 15 to 32) (C). Charged residues are denoted by white circles. Increasing hydrophobicity is denoted by increasing shading. The positions of the side chains are shown along a "regular" α-helix. For a right-handed helix with 3.6 residues per turn, rotation is clockwise as the polypeptide chain is followed from N to C, and the 5th residue ends up in a position exactly 40° clockwise relative to that of residue 1.

Defb14-1C^V(14-23) has very poor activity against both Gram-positive and -negative strains, whereas Defb14-1C^V(1-10) and Defb14-1C^V(6-17) retain some aspects of the antibacterial activity of the parent molecule. Both fragments are potent bactericides against the three Gram-negative strains tested; however, only Defb14-1C^V(6-17) is robust against the Gram-positive *S. aureus* strains. The deletion series allowed us to determine whether these fragments influence bactericidal activity within the context of the full-length molecule. We find that as the N-terminal deletions progress, the monomeric peptide MBC increases (indicating loss of activity), but the points at which significant MBC increase occurs vary between strains. The deletion series indicates that the critical amino acid residues required for bactericidal activity are located between residues 5 and 14 (concordant with the activity of fragments 1 to 10 and 6 to 17). Gram-positive strains MRSA J2918 and *S. aureus* ATCC 25923 are effectively killed by Defb14-1C^{V}Δ(6-17) but not Defb14-1C^{V}Δ(1-10). In agreement with this, the ability of the truncated peptides to kill *S. aureus* ATCC 25923 and MRSA J2918 reduces significantly with the deletion of residues 9, 10, and 11 (FFA). The charge of active Defb14-1C^{V}Δ(1-8) is the same (+8) as that of poorly active Defb141C^{V}Δ(1-11), with little change in hydrophobicity, suggesting that the difference in activity is indeed primarily sequence/structure based. This contrasts with work on hBD3, where charge and hydrophobicity have been found to influence antimicrobial activity (9).

Although amino acid residues 9 to 11 appear to be important in the killing of Gram-positive *S. aureus* strains, deletion of these residues results in little change to the monomeric MBC against Gram-negative *P. aeruginosa* PAO1 and *A. baumannii*. However, the subsequent deletion of RIR in Defb14-1C^V(1-14) results in a significant loss of activity. This decrease in sensitivity is not wholly dependent on the Gram status of the bacteria, since Gram-negative *E. coli* ATCC 25922 shows sensitivity to Defb14-1C^{V}Δ(1-11) in a pattern similar to that of Gram-positive *S. aureus* species.

These data again suggest that the primary amino acid sequence is important for bactericidal activity. This notion is supported by a similar study with hBD1 which revealed that antimicrobial activity against *E. coli* was affected mostly by amino acid substitutions of charged residues in the positively charged C-terminal region (15). These substitutions did not affect peptide topology, and the study concluded that it was the side chain changes that affected the antibacterial activity. Recently, it was reported that the bactericidal activity of α-defensin cryptdin 4 was attenuated when all the arginines were mutated to lysine. Llenado et al. (10) postulate that the guanidine group in arginine may allow stronger electrostatic charges and hydrogen bonding interactions, which may be important for electronegative bacterial cell membrane interactions. However, we have tested whether inclusion of the RIR sequence results in a peptide with bactericidal activity, but peptide Defb14-1C^V(12-23) with sequence RIRGGRAA VLNA is inactive as a bactericide at 20 μM against both *P. aeruginosa* and *S. aureus* (data not shown).

Multiple sequences contribute to bactericidal activity. *E. coli*, *P. aeruginosa* PAO1, and *A. baumannii* are killed effectively by peptide fragments Defb14-1C^V(1-10) and Defb14-1C^V(6-17). The deletion series monomer data support both of these fragments containing unique sequence that is important in the context of the peptide. Sequential deletion of amino acids 6, 7, and 8 and then 9, 10, and 11 (*E. coli*) and amino acids 9, 10, and 11 followed by 12, 13, and 14 (*P. aeruginosa*) results in an individual decrease in bactericidal potency. This suggests that there are two regions important to bactericidal activity, one in Defb14-1C^{V}1-10 and one in Defb14-1C^{V}6-17, so that only when both are deleted from the larger molecule is the activity very much reduced.

Supporting this is our previous finding that the sequence NTLQK in hBD3 (corresponding to KTLRK in Defb14) has been subjected to negative selection during primate evolution (19). This sequence is contained fully in Defb141C^V(1-10) and partially in Defb141C^V(6-17), suggesting that the region is important functionally. Interestingly, it is also this region (LRK) that appears to be critical in maintaining the salt insensitivity of the peptides against *P. aeruginosa*.

Dimeric structure retains activity. The bactericidal activity of the dimeric deletion peptides is more resistant to the deletion of amino acids than that of the monomeric forms up until the Defb14-1C^{V}Δ(1-14) fragment (against *S. aureus*) and deletion of RIR. This indicates that dimer structure, in addition to sequence, is important for bactericidal activity. The increased charge of dimeric molecules may implicate charge as a contributor to antibacterial activity; however, the inactivity of dimeric Defb14-1C^{V}Δ(1-14) implies that sequence and structure are more important than charge for this strain. In order to consider this further, we compare the charges and bactericidal activities for the various peptides against *P. aeruginosa* PAO1 (Fig. 1). Comparison of bactericidal activities between Defb14-1C^{V}Δ(1-23) and Defb14-1C^{V}Δ(1-17) dimers with the same charge (+5) and similar low hydrophobicities but vastly different bactericidal activities supports that sequence and structure are more important than charge.

The importance of dimeric structure is further supported by evidence that covalent dimeric defensins are more active bactericides than monomeric or noncovalent dimers (2, 4) and by the recent report by Antcheva et al. (1) which demonstrated that covalent dimerization of an artificial defensin could improve its antimicrobial activity.

Helical structure may affect function. We performed circular dichroism (CD) on an analogue of Defb14-1C^{V}Δ(1-23), which has poor antimicrobial activity identical to that of Defb14-1C^{V}Δ(1-23) but alanines at all the cysteine positions (20). When compared to the potent N-terminal peptide Defb14-1C^{V}(1-23), it is shown to have reduced helical propensity (21; data not shown). We also show by helical wheel projection that the helix-forming propensity of the N-terminal region is likely to be lost as the peptide deletions increase. This may be important since the formation of helical oligomers is implicated in the pore model of antimicrobial action. However, there is little difference in these projections for residues 12 to 29 and 15 to 32 to rationalize the lack of bactericidal activity against *P. aeruginosa* of the Defb14-1C^{V}Δ(1-14) monomer relative to that of Defb14-1C^{V}Δ(1-11). In addition, the activities found in these deletion peptides and mode of action may not reflect those present in the full-length disulfide stabilize molecule.

Of the peptides tested here, Defb14-1C^{V}(6-17) has the most potential as a short and effective bactericidal agent *in vitro*. We have, however, found that the bactericidal activities of the peptide fragments and parental β-defensin are inhibited by serum, although not by trypsin digestion (data not shown), which is also observed for hBD3 (12).

In conclusion, our results show that both structure and sequence are important for the antimicrobial activity of these β-defensin derivatives. We find that the activities of the N-terminal peptide fragments are supported by the N-terminal deletions, and we find that the precise sequences that confer bactericidal activity vary for different bacteria, showing selectivity at the amino acid level. We have shown that deletion of FFA and RIR is important for loss of activity against *S. aureus*, deletion of LRK for *E. coli* and *E. faecalis*, and deletion of RIR for *P. aeruginosa* and *A. baumannii*. We also show that, in all cases, covalent dimer peptides retain activity better than monomeric forms. Approaches aimed at determining the molecular basis of structure-activity relationships will greatly assist in the design of novel therapeutic agents.

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Short communication

Isoleucine/leucine[2] is essential for chemoattractant activity of β-defensin Defb14 through chemokine receptor 6

Christine Tyrrell[a,1], Martin De Cecco[b,1], Natalie L. Reynolds[a], Fiona Kilanowski[a], Dominic Campopiano[b], Perdita Barran[b], Derek Macmillan[c,1], Julia R. Dorin[c,*,1]

[a] MRC Human Genetics Unit, IGMM, Edinburgh EH4 2XU, Scotland, UK
[b] School of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, UK
[c] Department of Chemistry, Christopher Ingold Laboratories, University College London, WC1H 0AJ, UK

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ABSTRACT

β-Defensins are both antimicrobial and able to chemoattract various immune cells including immature dendritic cells and CD4 T cells through CCR6. They are short, cationic peptides with a highly conserved six-cysteine motif. It has been shown that only the fifth cysteine is critical for chemoattraction of cells expressing CCR6. In order to identify other residues essential for functional interaction with CCR6 we used a library of peptide deletion derivatives based on Defb14. Loss of the initial two amino acids from the Defb14-1C^{V} derivative destroys its ability to chemoattract cells expressing CCR6. As the second amino acid is an evolutionarily conserved leucine, we make full-length Defb14-1C^{V} peptides with substitution of the leucine[2] for glycine (L2G), lysine (L2K) or isoleucine (L2I). Defb14-1C^{V} L2G and L2K and are unable to chemoattract CCR6 expressing cells but the semi-conservative change L2I has activity. By circular dichroism spectroscopy we can see no evidence for a significant change in secondary structure as a consequence of these substitutions and so cannot attribute loss of chemotactic activity with disruption of the N-terminal helix. We conclude that isoleucine/leucine in the N-terminal α-helix region of this β-defensin is essential for CCR6-mediated chemotaxis.

1. Introduction

β-Defensins are believed to be important components in innate immunity. They are a multigene family which has ~40 members spread over five genomic loci in human (Schutte et al., 2002). Defensins have a canonical six-cysteine motif but the remaining sequence of these short peptides is not highly conserved. *In vitro* they possess a strong, broad spectrum antimicrobial action and also have been shown to be chemoattractant for various immune cells including CD4 memory T helper cells and immature (but not mature) dendritic cells (iDC) through chemokine receptor 6 (CCR6) (Yang et al., 1999). We also have shown that the murine Defb8 and a five cysteine peptide (encoded by the *Defr1* allele of *Defb8*) are chemoattractant for CD4^{+} T cells and iDC but not mature DC and not through CCR6 (Taylor et al., 2009). β-Defensins are therefore a promiscuous family of ligands. Candille et al. (2007) have shown that human β-defensins can interact with melanocortin receptors *in vitro*, and *in vivo* over-expression of the dog *CBD103* gene (orthologue to *DEFB103*) in transgenic mice causes black hair colour through binding to MC1R. Recently, increased copy number of the β-defensins at the chromosome 8 locus has been associated with increase risk of psoriasis (Hollox et al., 2008).

Human β-defensin 3 (hBD3, *DEFB103*) has multiple functions (Dhople et al., 2006) and *Defb14* is the mouse orthologue (Semple et al., 2003) and has similar antimicrobial properties (Taylor et al., 2008; Rohrl et al., 2007; Hinrichsen et al., 2008). Defb14 mature peptide, with the full complement of the six-cysteine residues characteristic of a β-defensin, can chemoattract cells expressing the chemokine receptor CCR6 and monocytes (Taylor et al., 2008). Wu et al. (2003) elegantly used directional disulfide bonding to demonstrate that the disulfide connectivities influenced the chemoattractant potency of hBD3. We have shown (Taylor et al., 2008) that hBD3 or Defb14 peptides with a single cysteine at position V of the six-cysteine motif (–C^{I}–C^{II}–C^{III}–C^{IV}–C^{V}C^{VI}–) where the cysteines are interspersed with a variable number of amino acid residues, are as active as the peptide with the native connectivity (C$_{I}$–C$_{V}$, C$_{II}$–C$_{IV}$, and C$_{III}$–C$_{VI}$). Peptide with no cysteine (cysteines replaced with alanines) or peptide fragments are not active as chemoattractants, which implied that both structure and residue side chains were essential for functional interaction with the CCR6 receptor.

* Corresponding author at: MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland, UK. Tel.: +44 131 467 8411; fax: +44 131 467 8456.
E-mail address: julia.dorin@hgu.mrc.ac.uk (J.R. Dorin).
[1] These authors contributed equally to this study.

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We show here that N-terminal deletions of the peptide – even deletion of only the first two N-terminal residues – eliminate the chemoattractant ability of the peptide. Further analysis revealed that the ability to chemoattract CCR6-expressing cells is dependent on the residue side chain rather than the helicity of the N-terminal region of the peptide.

2. Materials and methods

2.1. Peptide synthesis and purification

All peptides were chemically synthesized by standard solid-phase methodology. Defb14-1C^{V} and derivatives were made "in house" using automated peptide synthesis. This was carried out on an Applied Biosystems model 433A peptide synthesizer using Rink amide AM resin for peptide amides, preloaded NovaSyn®TGT resin for peptide acids, and Fmoc amino acids from Novabiochem. All truncated Defb14-1C^{V} (Fig. 1) were synthesised with an acetyl group at the N terminus, rather than the free amino group to best represent the conformation of the terminal amino acid. LC-mass spectra confirming identity and purity were obtained on a Micromass Quattro LC mass spectrometer. Semi-preparative HPLC was performed using a Phenomenex Luna C18 column and a gradient of 5–95% acetonitrile (containing 0.1% trifluoroacetic acid) over 45 min (flow rate of 3.0 ml/min). All other chemical reagents were obtained from Aldrich. Automated solid-phase peptide synthesis was carried out on a 0.05-mmol scale using 0.5 mmol of each Fmoc amino acid per coupling reaction and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole as coupling reagents. Coupling time was 0.5 h. Peptide products were cleaved from the resin with 95% trifluoroacetic acid, 2.5% ethanedithiol, 2.5% water for 3 h; the resin was filtered off and washed with trifluoroacetic acid; and filtrate was poured into diethylether (10 volumes). Following centrifugation (3000 rpm, 15 min) the precipitate was resuspended in ether (5 volumes) and recentrifuged (3000 rpm, 15 min). The crude peptides were dissolved in water and loaded directly onto a semi-preparative HPLC column. Peptide fractions were identified by mass spectrometry and lyophilized.

2.2. Chemotaxis assay

The migration of CCR6-transfected human embryonic kidney HEK293 cells was assessed with a microchemotaxis chamber technique as described (Taylor et al., 2009). Briefly, peptides were serially diluted in RPMI medium containing 1% BSA. Dilutions or medium alone were dispensed into the lower wells of the chamber. CCL20 (Peprotech EC, London, UK) was used as a positive control at the same concentrations. A 10 μm polycarbonate filter (Receptor Technologies, Leamington Spa, UK), coated with 5 μg/ml human type VI collagen (Sigma, Dorset, UK) was placed over the lower chamber. 100,000 cells per well were then added to the upper portion of the chamber. Each dilution was tested in triplicate and experiments were carried out a minimum of three times. Medium alone was used as a control. CCL20 was obtained from Peprotech EC (London, UK). Migratory Index was calculated as compared to control values. Data was analysed by one-way ANOVA with post-test when $p < 0.01$. Chemotactic activity was determined as the concentration of test compound at which the highest migratory index was achieved.

2.3. Minimum bactericidal concentration (MBC assays)

These assays were carried out as previously described (Taylor et al., 2008). Briefly, test organisms were grown to mid-logarithmic phase in Iso-Sensitest broth (Oxoid) growth medium and then diluted to $1–5 \times 10^6$ colony-forming units/ml in 10 mM potassium phosphate containing 1% (v/v) Iso-Sensitest broth, pH 7.4. Different concentrations of test peptide were incubated in 100 μl of cells ($1–5 \times 10^5$ colony-forming units) at 37 °C for 3 h. 10-Fold serial dilutions of the incubation mixture were spread on Iso-Sensitest plates and incubated at 37 °C, and the colony-forming units were determined the following day. The minimum bactericidal concentration is the concentration of peptide where we observed >99.99% killing of the initial inoculum. All assays were performed in duplicate and repeated on four independent occasions. The minimum bactericidal concentration was obtained by taking the mean of all of the results, and experimental errors were within one doubling dilution.

2.4. Circular dichroism spectroscopy

Peptide solutions were prepared at an approximate concentration of 200 μg/ml in: (i) 10 mM ammonium acetate (Sigma), pH 6.8, and (ii) 50% water, 50% 2,2,2-trifluoroethanol (TFE) (Aldrich). CD spectra were recorded at 20 °C using a Jasco J-810 spectropolarimeter. Five scans were acquired for each sample at a rate of 10 nm min^{-1} and averaged to give a final sample spectrum. A 'blank' buffer CD spectrum, acquired in precisely the same way, was subtracted from each final sample spectrum. The concentration of each peptide solution was determined subsequently by assaying with bicinchoninic acid (Smith et al., 1985) (Pierce) and the mean residue ellipticity (θ_λ) was calculated at each wavelength (λ). The CDSSTR algorithm (Johnson, 1999) on the DICROWEB server (Whitmore and Wallace, 2008) was used to help assign secondary structure.

3. Results and discussion

3.1. Chemoattractant activity is lost following deletion or replacement of Leucine^2 of Defb14-1C^{V}

Fig. 1a shows the ability of N-terminal deletions of Defb14-1C^{V} to chemoattract cells expressing human CCR6. Supplementary Fig. 1 shows flow cytometry analysis to confirm that these cells strongly express CCR6 with an antihuman CCR6 antibody compared to the signal obtained with an isotype control. Hek cells not expressing CCR6 do not show any chemoattraction to these defensins (data not shown and Taylor et al., 2008). Only the parent peptide Defb14-1C^{V} shows a typical bell-shaped curve and migratory indices are significantly different to media alone. Loss of the first two (phenylalanine and leucine) amino acids of Defb14-1C^{V} results in loss of chemoattraction (Fig. 1a). Rohrl et al. (2007) describe a recombinant mBD14-Ig fusion protein acting as a chemoattractant for cells expressing murine CCR6, which perhaps implies the C-terminal end of the molecule can support some structural change without altering its ability to interact functionally with CCR6. The results in Fig. 1 presented here demonstrate that losing the first (phenylalanine) and second (leucine) amino acids results in the complete loss of the CCR6-mediated chemoattractant activity of the peptide. Thus one of these residues appears to be essential for direct interaction with the receptor, or alternatively directs the peptide to achieve a conformation which presents other residues such that they can bind successfully to the receptor. This postulated conformation may only occur in the presence of the receptor. hBD3, the human orthologue of Defb14 (which has comparable activity to chemoattract these CCR6 expressing cells) has glycine (G) and isoleucine (I) as the first two amino acids of the mature peptide where Defb14 has phenylalanine (F) and leucine (L). Leucine to isoleucine is a highly conservative residue change both being hydrophobic residues and in other mammals this second residue is also conserved. In Chinchilla, Cow, Opossum, Pig and Dog the second residue is either isoleucine or leucine. The first residue is however less conserved,

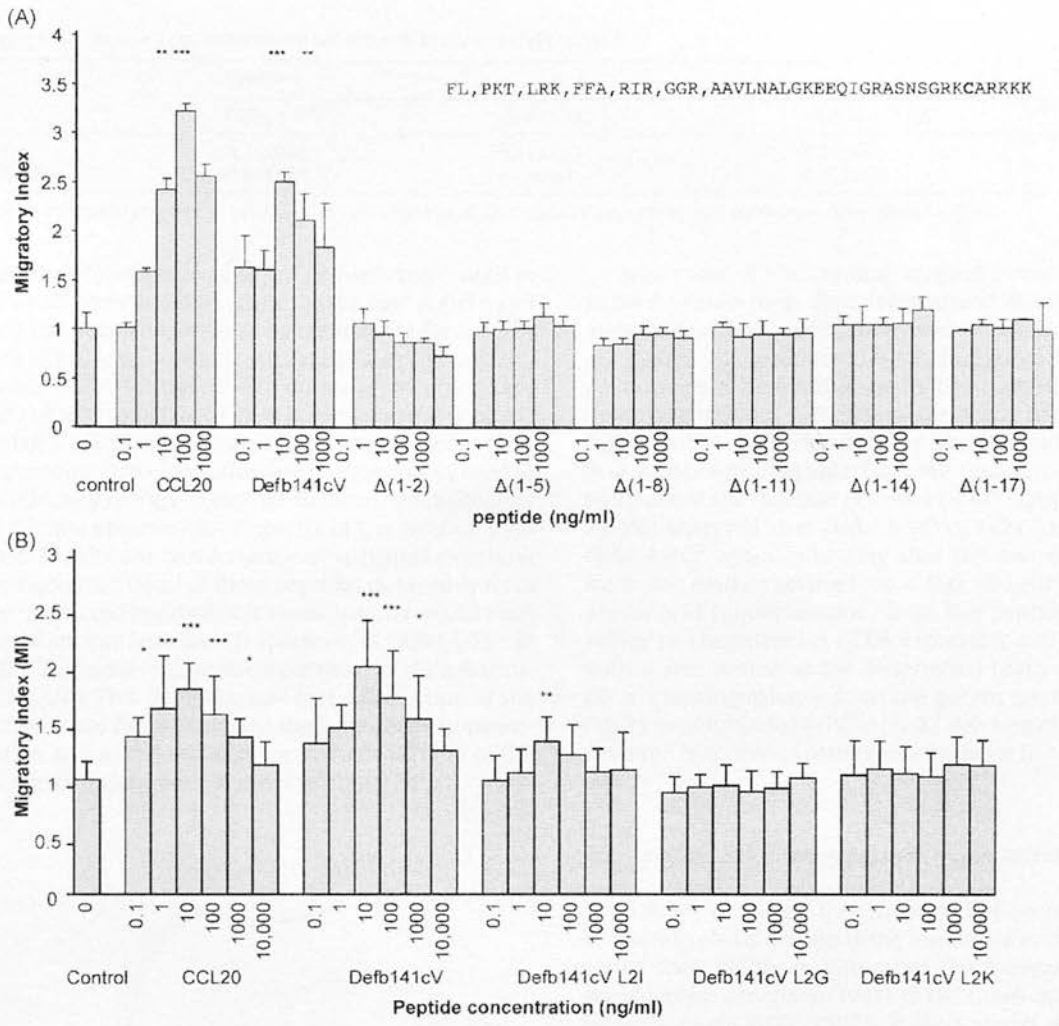

Fig. 1. Leucine at position 2 is essential for the chemoattractant activity of Defb14-1C^{V} against human embryonic kidney cells expressing CCR6. (A) The graph shows the number of HEK293 cells expressing the human chemokine CCR6 cells that migrate toward the test deletion peptides (A) or substitution peptides (B). The migratory index (ratio of the number of cells per high power field with peptide to number of cells media alone) is given. CCL20 (Mip3α) is the chemokine ligand of CCR6 and Defb14-1C^{V} is the active parent peptide. Each assay was repeated at least three times, and three fields of view were taken for each experiment. Numerical data are means ± S.D. Above graph (A) is the peptide sequence in single letter code of Defb14-1C^{V} with commas indicating the deletion points. * indicates difference from medium alone control $p > 0.05$, $^{**}p > 0.01$, $^{***}p > 0.001$.

a G in Human, Chinchilla, Cow and Dog but an F in Mouse, R (arginine) in Pig, and W (tryptophan) in Opossum. This suggests that the second highly conserved residue might be the essential factor for this β-defensin to functionally interact with CCR6 to induce chemoattraction. In addition, hBD2, another β-defensin shown to have ability to chemoattract through CCR6 (Yang et al., 1999), has isoleucine as its second amino acid.

In order to determine if the loss of chemoattractant activity of Defb14-1C^{V}Δ(1–2) was the consequence of the loss of leucine, and not as result of length reduction, we created three substitution mutants where the second hydrophobic leucine residue was replaced either with the charged residue lysine, the hydro-neutral glycine or the semi-conservative change to isoleucine. Fig. 1b shows that the peptide Defb14-1C^{V} with replacement of the leucine to lysine (L2K) was unable to chemoattract the CCR6 expressing cells and neither was Defb14-1C^{V} L2G. Defb14-1C^{V} L2I had a reduced ability to chemoattract the CCR6 cells with a Migratory Index (MI) of 1.4 at 10 ng/ml. This result indicates that the residue side chain at the second position of the N-terminal region is indeed important for the peptide's ability to interact functionally with CCR6.

The N-terminal region of β-defensins forms an α-helix and so in order to determine whether the above mutations result in a change in secondary structure of the molecule we carried out circular dichroism spectroscopy (CD).

3.2. *Reduced chemotactic activity is not due to impairment of the N-terminal helix*

We investigated the secondary structures of the substitution mutants by CD and compared the spectra to those of the active full-length parent peptide Defb14-1C^{V}. The CD spectrum of Defb14-1C^{V} in aqueous solution (Fig. 2a) shows an absorption minimum at ~201 nm that is typical of a peptide whose secondary structure is a mixture of β-sheet or β-turn and random coil. This is in good qualitative agreement with published CD spectra of other β-defensins, including Defb14 orthologue hBD3 (Kluver et al., 2005; Liu et al., 2008). In buffer, Defb14-1C^{V} exhibits a slightly more intense absorption minimum than that of the other peptides but was found to contain the same proportions of secondary structure as all of the other peptides according to analysis by the CDSSTR algorithm (Johnson, 1999).

HBD1 and 2 interact with CCR6 to induce cell migration but with less affinity than hBD3. A comparison of the crystal structures of CCL20/MIP-3α (the chemokine ligand of CCR6) and β-defensins has revealed some structural similarities despite a lack of linear

Table 1
MBC values for Defb14-1C^{V} Leucine 2 substitutions are not different to the parental peptide.[a].

Bacterium	Peptide			
	Defb14-1C^{V}	Defb14-1C^{V} L2I	Defb14-1C^{V} L2G	Defb14-1C^{V} L2K
P. aeruginosa PAO1	0.2 (±0.0)	0.5 (±0.1)	0.3 (±0.0)	0.3 (±0.0)
S. aureus ATCC 25923	0.5 (±0.0)	1.1 (±0.2)	0.9 (±0.2)	0.8 (±0.2)

[a] The concentration of peptide required to kill 99.99% of the initial inoculum of either *P. aeruginosa* or *S. aureus* was determined (±SEM).

sequence similarity (Hoover et al., 2002). The Asp^{4}–Leu^{9} motif in HBD2 and Asp^{1}–Val^{6} motif in HBD1 resemble the Asp^{5}–Leu^{8} motif of CCL20, which has been suggested to be responsible for specific interaction with CCR6, providing a structural basis for the capacity of β-defensins and CCL20 to interact with the same receptor. These regions include the N-terminal α-helix of the defensin structure.

The addition of 2,2,2-trifluoroethanol (TFE) to an aqueous peptide solution can induce helix formation and it is commonly used to evaluate the propensity of a given peptide to form a helix (Kelly et al., 2005). The minima observed in all spectra of the deletion peptides at 208 and 222 nm are characteristic of α-helical structure, which strongly suggests that all of these peptides can readily form α-helices under increased hydrophobic conditions, as would exist at or in a bacterial membrane. The CD spectrum of Defb14-1C^{V} in 50% water, 50% TFE is indistinguishable from those of the substitution mutants (Fig. 2b). This demonstrates that substitution of the leucine at position 2 has not diminished the helix-forming potential of the peptide and so the reduced chemotactic activity of the mutants is not due to impairment of the N-terminal helix.

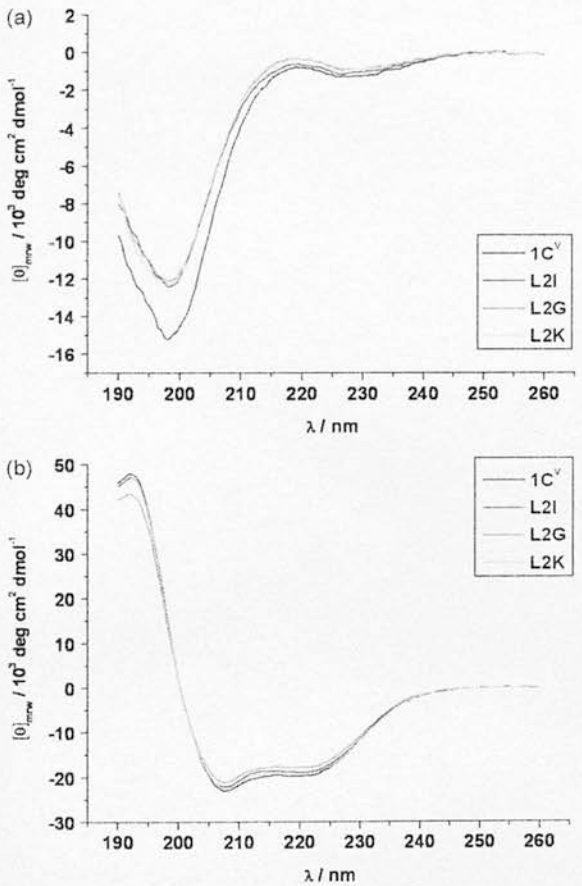

Fig. 2. Substitution of leucine at position 2 does not significantly change the secondary structure of Defb14-1C^{V}. (a) Spectra of Defb14-1C^{V} and substitution peptides recorded in 10 mM ammonium acetate. (b) Spectra of the same peptides recorded in 50% water and 50% 2,2,2-trifluoroethanol (TFE). Each spectrum has been normalised for concentration as described in the text.

Inspection of the nuclear magnetic resonance structures of hBD3 reveals a long, disordered amino terminal region immediately before the α-helix, composed of residues 10–14 (Schibli et al., 2002). The predominantly hydrophobic nature of the first 9 amino acids of both hBD3 and Defb14 suggests that a longer helix (involving residues 1–9) may be formed at the N terminus in more hydrophobic conditions such as those found in a membrane or at a protein–protein interface. We also analysed the secondary structure of the deletion peptides by CD (supplementary data Fig. 2). We observed that Defb14-1C^{V}Δ(1–2) has similar spectra to Defb14-1C^{V} again indicating that the loss of chemotactic ability is not easily explained by a loss of helicity. Loss of the first amino acid (phenylalanine) from the peptide does not alter its ability to chemoattract CCR6 expressing cells when synthesised with a free amine at the N-terminal (data not shown) and its CD is indistinguishable from the parent peptide (supplementary Fig. 2). Beyond Defb14-1C^{V}Δ(1–2), deletion of further amino acids from the N-terminus results in a decrease in helix-forming potential.

3.3. *Leucine^{2} substitution peptides retain bactericidal activity*

In order to ensure that the substitution peptides were active as antimicrobials despite being less active as chemoattractants we tested their ability to kill either Gram-negative bacterial strain *Pseudomonas aeruginosa* PAO1 or the Gram-positive strain *Staphylococcus aureus* ATCC 25923. Table 1 shows that the substitution peptides were still potent bactericidals and not significantly different from the parental peptide in their ability to kill the bacteria tested.

4. Conclusion

We show here that deletion or substitution of the second amino acid of the Defb14-1C^{V} peptide results in a loss of chemoattractant activity. This loss of function is not the result of any observable change in the secondary structure of the free peptide. It is more likely a consequence of the conserved leucine at position 2 of the mature peptide being part of the ligand motif required for interaction with CCR6. Isoleucine can partially substitute for leucine to allow chemoattraction of CCR6-expressing cells. Thus residues at both the N-terminal (L/I at residue 2) and C-terminal regions (C at residue 40) of Defb14 are essential for a chemotactically competent peptide. β-Defensins are known to be important in host defence and as a link between innate and adaptive immunity, thus identification of residues essential for chemoattraction of CD4 T cells and immature DC is important for understanding the functional consequence of mutation and designing therapeutics with defined action.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.11.025.

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"So long and thanks for all the fish"

Douglas Adams